ORIGINAL SUBMISSION

Sent via Fedex

October 12, 2011

Dr. Mary Ditto
Office of Food Additive Safety (HFS-200)
Center for Food Safety & Applied Nutrition
U.S. Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

GRAS Notice for *Lactobacillus reuteri* NCIMB 30242

Dear Dr. Ditto:

In accordance with 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting in triplicate, as the Notifier [Micropharma Ltd. 141 avenue du President Kennedy 5th Floor, UQAM - Biological Sciences Building Unit 5569 Montreal, Quebec, Canada], a Notice of the determination, on the basis of scientific procedures, that *Lactobacillus reuteri* NCIMB 30242 distributed by Micropharma, as defined in the enclosed documents, is GRAS under specific conditions of use as an ingredient in multiple food categories, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. Information setting forth the basis for the GRAS determination, includes a comprehensive summary of the data available that has been reviewed by an independent panel of experts (the Expert Panel) qualified by scientific training and experience to evaluate the safety of *Lactobacillus reuteri* NCIMB 30242 in traditional food products.

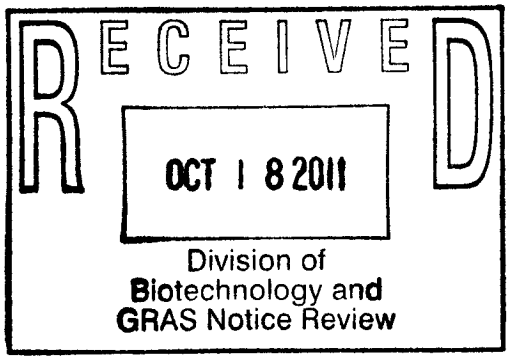
Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

Christopher Wahl, MD, MBA
Director of Business Development
Micropharma Ltd.

Lactobacillus reuteri NCIMB 30242
GRAS Notice



Prepared for:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Prepared by:

Micropharma Ltd.
141 avenue du President Kennedy
5th Floor, UQAM - Biological Sciences Building
Unit 5569
Montreal, Quebec, Canada
H2X 3Y7

October 12, 2011

Lactobacillus reuteri NCIMB 30242 GRAS Notice

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Lactobacillus reuteri NCIMB 30242 GRAS Notice

I. GRAS EXEMPTION CLAIM

A. Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)] (U.S. FDA, 1997)

As defined herein, *Lactobacillus reuteri* NCIMB 30242 (*L. reuteri* NCIMB 30242), has been determined by Micropharma Ltd. (Micropharma) to be Generally Recognized as Safe (GRAS), consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, and on the consensus opinion of an independent panel of Experts qualified by scientific training and expertise to evaluate the safety of *L. reuteri* NCIMB 30242 under the conditions of intended use in food. Therefore, the use of *L. reuteri* NCIMB 30242 in food as described herein is exempt from the requirement of premarket approval (Section 409 of the *Federal Food, Drug and Cosmetic Act*).

Signed,

(b) (6)

Christopher Wahl, MD, MBA
Director of Business Development
Micropharma Ltd.

October 12, 2011
Date

B. Name and Address of Notifier

Micropharma Ltd.
141 avenue du President Kennedy
5th Floor, UQAM - Biological Sciences Building
Unit 5569
Montreal, Quebec, Canada
H2C 3Y7

C. Common Name of the Notified Substance

Lactobacillus reuteri NCIMB 30242

D. Conditions of Intended Use in Food

Micropharma intends to market *L. reuteri* NCIMB 30242, as a food ingredient for use in multiple food and beverage categories at use levels ranging from 0.17 to 3.25% as described in Table I.D-1.

Table I.D-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *Lactobacillus reuteri* NCIMB 30242 in the United States (2003-2006 NHANES Data)

Food Category	Proposed Food-Uses	*L. Reuteri* Use Level (g/serving)	Serving Size (g or mL)[c]	*L. Reuteri* Use-Level (%)[b]
Beverages and Beverage Bases	Meal Replacement Beverages	$1x10^{10}$	240	0.17
Breakfast Cereals	Ready-to-Eat Breakfast Cereals	$1x10^{10}$	15 (Puffed)	2.67
			30 (Regular)	1.33
			55 (Biscuit-Type)	0.73
Cheeses	Cream Cheese[c]	$3.3x10^{8}$	30	0.43
	Natural Cheese[c]	$3.3x10^{8}$	30	0.43
	Processed Cheese and Spreads[c]	$3.3x10^{8}$	30	0.43
Dairy Product Analogs	Soy-Based Beverages	$1x10^{10}$	240	0.17
Fats and Oils	Butter	$3.3x10^{8}$	15	0.87
	Fat-Based Sauces	$3.3x10^{8}$	30	0.43
	Margarine[c] and Margarine-like Spreads	$3.3x10^{8}$	15	0.87
	Mayonnaise[c] and Mayonnaise-Type Dressings	$3.3x10^{8}$	15	0.87
	Salad Dressings[c]	$3.3x10^{8}$	30	0.43
	Vegetable Oils	$3.3x10^{8}$	15	0.87
Frozen Dairy Desserts	Frozen Novelties and Frozen Milk Desserts	$1x10^{10}$	120	0.33
	Frozen Yogurt	$1x10^{10}$	120	0.33
	Ice Cream[c]	$1x10^{10}$	120	0.33
			240 (sundaes)	0.17
Grain Products and Pastas	Cereal and Granola Bars	$1x10^{10}$	40	1.00
	Energy, Meal Replacement, and Fortified Bars	$1x10^{10}$	40	1.00
Milk Products	Fermented Milks (plain)[c]	$1x10^{10}$	240	0.17
	Flavored Milk, Milk Drinks, and Mixes	$1x10^{10}$	120 (eggnog)	0.33
			240	0.17
	Milk-Based Meal Replacement Beverages	$1x10^{10}$	240	0.17
	Sour Cream[c]	$3.3x10^{8}$	30	0.43
	Yogurt[c]	$1x10^{10}$	225	0.18
	Yogurt Drinks[d]	$1x10^{10}$	240	0.17

Table I.D-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *Lactobacillus reuteri* NCIMB 30242 in the United States (2003-2006 NHANES Data)

Food Category	Proposed Food-Uses	*L. Reuteri* Use Level (g/serving)	Serving Size (g or mL)[c]	*L. Reuteri* Use-Level (%)[b]
Processed Fruits and Fruit Juices	Fruit Juices	$1x10^{10}$	240	0.17
Soft Candy	Chocolate Confectionary	$3.3x10^{8}$	40	0.33
Sugar Substitutes	Sugar Substitutes	$3.3x10^{8}$	4	3.25

[a] Serving sizes were based on Reference Amounts Customarily Consumed (RACC) per Eating Occasion in the U.S. CFR (21 CFR §101.12) (U.S. FDA, 2011a).
[b] Based on estimate that 400 mg of lyophilized *L. reuteri* powder contains $1x10^{10}$ CFU
[c] These food-uses represent non-standardized food products; however, in order to obtain a conservative intake estimate, surrogate codes for the standardized food products were chosen.
[d] No food codes were identified for yogurt drinks; therefore, surrogate codes of fruit smoothie drinks were used to represent the food codes in this category.

E. Basis for the GRAS Determination

Pursuant to 21 CFR §170.30, *L. reuteri* NCIMB 30242, has been determined by Micropharma to be GRAS on the basis of scientific procedures (U.S. FDA, 2011a). This GRAS determination is based on data generally available in the public domain pertaining to the safety of *L. reuteri* NCIMB 30242 for use in food, as discussed herein and in the accompanying documents, and on a consensus among a panel of Experts[1] who are qualified by scientific training and experience to evaluate the safety of *L. reuteri* NCIMB 30242 as a component of food.

F. Availability of Information

Data and information that serve as the basis for this GRAS Notice will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of Micropharma located at the following address:

Micropharma Ltd.
141 avenue du President Kennedy
5th Floor, UQAM - Biological Sciences Building
Unit 5569
Montreal, Quebec, Canada
H2C 3Y7

Should the FDA have any questions or additional information requests regarding this Notice, Micropharma also will supply these data and information.

[1] The Expert Panel consisted of Dr. Michael W., Pariza Ph.D. (University of Wisconsin), Dr. Stephen L., Taylor Ph.D. (University of Nebraska), and Dr. Gary M., Williams, M.D. (New York Medical College). A copy of the Expert Panel summary is located in Appendix A and is titled "Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Use of *Lactobacillus reuteri* NCIMB 30242 as a Food Ingredient in Multiple Food Categories".

II. DETAILED INFORMATION REGARDING THE IDENTITY OF THE SUBSTANCE

A. Identity

Common Name: *Lactobacillus reuteri* NCIMB 30242

Trade Name: *Lactobacillus reuteri* Cardioviva™

Taxonomic Lineage:

Kingdom: *Bacteria*
Phylum: *Firmicutes*
Class: *Bacilli*
Order: *Lactobacillales*
Family: *Lactobacillaceae*
Genus: *Lactobacillus*
Species: *Lactobacillus reuteri*
Strain: NCIMB[2] 30242; Cardioviva™

B. History of *Lactobacillus reuteri* NCIMB 30242

Although the original commensal species or environment for which *L. reuteri* NCIMB 30242 was isolated is not known, available evidence suggests that the organism was originally obtained from a porcine source (Lee *et al.*, 2009a). Following in-house research of several microorganisms for their ability to remove cholesterol from culture media, and normalize cholesterol levels in hypercholesteremic rodent models, an *L. reuteri* isolate was selected for development for food use by cardiovascular health conscious consumers. This novel strain has been given the trade name *L. reuteri* Cardioviva™ and is currently deposited in the National Collection of Industrial and Food Bacteria international culture collection under NCIMB 30242. The species and strain identity have been characterized using the most current phenotypic and genetic techniques (Sections II.C and II.D below).

C. Phenotypic Characterization

Lactobacilli are rod or coccobacilli shaped Gram-positive, non-spore-forming micro-organisms. They are catalase negative, fermentative, microaerophylic and chemo-organotropic, and have limited metabolic capacity requiring a nutrient rich media for growth. The microorganisms can be found in a variety of environments including food (dairy products, fermented meat, sour dough, vegetables, fruits, and beverages), and in respiratory, gastrointestinal, and genital tracts of humans and animals (Felis and Dellaglio, 2007).

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[2] NCIMB = The National Collection of Industrial, Marine and Food Bacteria

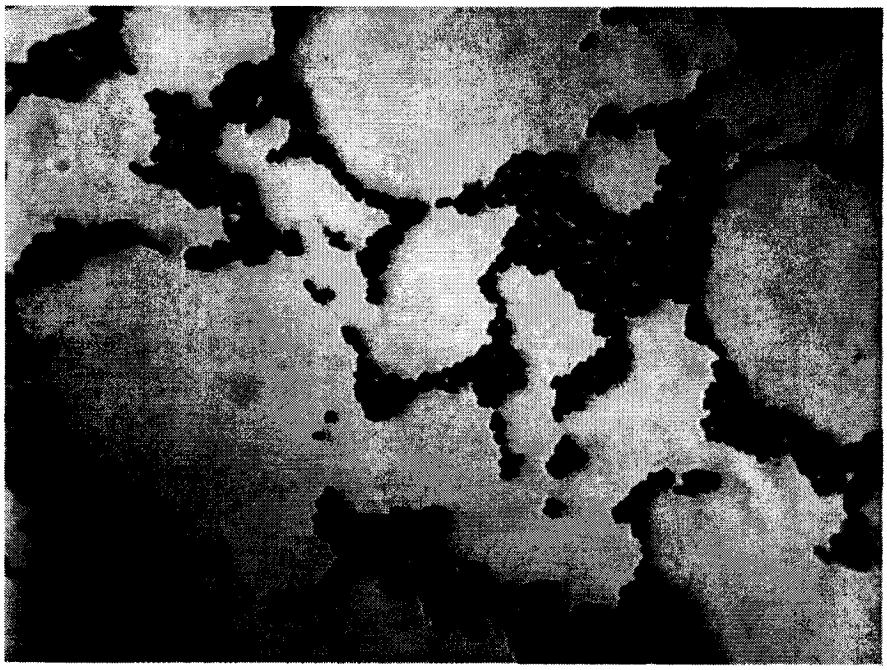

Figure 2.3-1 Photomicrograph of Gram-positive bacillus *Lactobacillus reuteri* (NCIMB 30242) show to be a facultative anaerobe and at 400x magnification.

(i) Carbohydrate Fermentation Profile

When tested with the API 50 CHL[3] (bioMérieux Clinical Diagnostics) test system according to the manufacturer's suggested protocol, *L. reuteri* NCIMB 30242 showed a fermentation profile identifying the organism as *Lactobacillus fermentum* (Table II.C-1). This was expected as the fermentation profile of *L. reuteri* is known to be similar to that of *L. fermentum* and the APIweb software does not distinguish between the 2 species. This fermentation pattern has been shown to be reproducible across multiple fermentation batches, but is highly dependent on the growth conditions used to produce the inoculums.

Table II.C-1 Carbohydrate Fermentation Profile of *Lactobacillus reuteri* NCIMB 30242 by API 50 CHL

Substrate	Result	Substrate	Result
Negative control	-	Esculin ferric citrate	-
Glycerol	-	Salicin	-
Erythritol	-	D-Cellobiose	-
D-Arabinose	-	D-Maltose	+
L-Arabinose	+	D-Lactose	+
D-Ribose	±	D-Melibose	+

[3] The API test system is composed of a plastic strip of microtubes containing dehydrated biochemicals that produce colorimetric reactions during fermentation, and permits the study of the microbial metabolism of 49 carbohydrates.

Table II.C-1 Carbohydrate Fermentation Profile of *Lactobacillus reuteri* NCIMB 30242 by API 50 CHL

Substrate	Result	Substrate	Result
D-Xylose	+	D-Saccharose	+
L-Xylose	-	D-Trehalose	-
D-Adonitol	-	Inulin	-
Methyl-βD-xylopyranoside	-	D-Melezitose	-
D-Galactose	+	D-Raffinose	+
D-Glucose	+	Amidon	-
D-Fructose	-	Glycogen	-
D-Mannose	-	Xylitol	-
L-Sorbose	-	Gentibiose	-
L-Rhamnose	-	D-Turanose	-
Dulcitol	-	D-Lyxose	-
Inositol	-	D-Tagatose	-
D-Mannitol	-	D-Fucose	-
D-Sorbitol	-	L-Fucose	-
Methyl-αD–mannopyroside	-	D-Arabitol	-
Methyl-αD-glucopyranoside	-	L-Arabitol	-
N-Acetylglucosamine	-	Potassium gluconate	-
Amygdalin	-	Potassium 2-ketogluconate	-
Arbutin	-	Potassium 5-ketogluconate	-

(ii) Enzymatic Profile

The enzymatic profile of the strain was assessed using APIzym test strips (bioMérieux Clinical Diagnostics) (Table IIC-2). The APIzym system is a rapid semiquantitative which allows the detection of 19 enzymatic reactions. Unlike API CHL 50 test strips a classification database for identification of the bacteria does not exist. The results of the assay are dependent on the growth conditions from which the bacteria were harvested and the incubation conditions used for the test. Table II.C-2 shows several enzymatic activities that are absent from *L. reuteri* NCIMB 30242 which agree with the carbohydrate usage pattern established by the API CHL50 test. One such example is that the APIzym test indicates that the strain lacks α-mannosidase activity and the API 50 CHL test indicates that the strain does not metabolize mannose.

Table II.C-2 Enzymatic Profile of *Lactobacillus reuteri* NCIMB 30242 by APIzym

Enzyme tested	Result
Alkaline phosphatase	-
Esterase (C4)	+
Esterase lipase (C8)	+
Lipase (C14)	-
Leucine arylamidase	+++

Table II.C-2 Enzymatic Profile of *Lactobacillus reuteri* NCIMB 30242 by APIzym

Enzyme tested	Result
Valine arylamidase	+
Cystine arylamidase	+
Trypsin	-
α-Chymotrypsin	-
Acid phosphatise	+
Naphthol-AS-BI-phosphohydrolase	+
α-Galactosidase	+++
β-Galactosidase	++
β-Glucuronidase	+/-
α-Glucosidase	++
β-Glucosidase	-
N-Acetyl-β-glucosaminidase	-
α-Mannosidase	-
α-Fucosidase	-

D. Genotypic Characterization

(i) Species Characterization and Genome Sequencing

Using a shotgun sequencing method, Micropharma has conducted a partial sequence of the whole genome of *L. reuteri* NCIMB 30242. Shotgun sequencing and de novo assembly was conducted at McGill University & Génome Québec Innovation Centre. Almost 230,000 reads were generated in over 79 Mb of sequencing data. The median read length for the region was 399 bp. From the assembly, 91% of the reads were fully assembled to yield 112 large contigs (>500 bp). The size of the genome was estimated at 1.78 Mb and the depth of sequencing coverage was at least 40x. The contigs generated at the Innovation Centre were then submitted to the RAST server (Aziz *et al.,* 2008) for annotation. Analysis of the annotated sequence was conducted using SEED Viewer (Overbeek *et al.,* 2005; version 2.0).

Using Basic Local Alignment Search Tool (BLAST), the gene sequence data of the entire 16S RNA gene was compared to the GenBank database of publically deposited 16S RNA gene sequences. A concise alignment (≥99%) of the 16S RNA sequence with the species *L. reuteri* was shown.

(ii) Strain Characterization

A DNA fingerprint profile of *L. reuteri* NCIMB 30242 was produced to facilitate strain identification of the organism. Amplified fragment length polymorphism (AFLP) analysis was used, a technique involving whole genome DNA fingerprinting *via* the selective amplification of restriction fragments (Vos *et al.*, 1995). Validation and conduct of the AFLP analyses was

conducted by by a qualified third-party expert (BCCM/LMG, Gent, Belgium). As shown below in Figure II.D-1, dendrogram analysis of the AFLP fingerprint profiles indicated that the AFLP analyses method was reproducible, and specific for identification of *L. reuteri* NCIMB 30242 This method is therefore suitable for quality control and post-market surveillance monitoring of *L. reuteri* NCIMB 30242.

In addition, a comparison of the AFLP profile with an internal database of AFLP DNA fingerprints of the lactic acid bacteria taxa (including bifidobacteria) as currently available in the BCCM/LMG database provided further confirmation, in addition to the 16S RNA alignment, for the identity of the organism under the species *L. reuteri.*

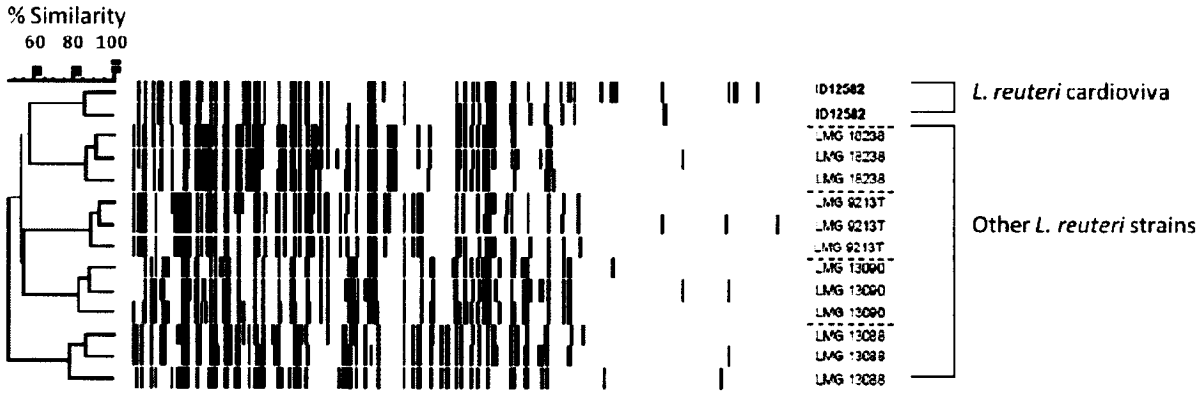

Figure II.D-1 AFLP Profile of *Lactobacillus reuteri* NCIMB 30242 Strain I.D. number with T represents the *L. reuteri* type strain

E. Manufacturing Information

(i) Fermentation of Commercial Cultures

Lactobacillus reuteri NCIMB 30242 is produced using traditional fermentation methods through contract manufacturers. Currently, the strain is manufactured at Chr Hansen (Denmark) using current Good Manufacturing Practices (cGMP). The master culture that is used to produce *L. reuteri* NCIMB 30242 is maintained at the culture bank of The National Collection of Industrial, Marine and Food Bacteria (NCIMB; Aberdeen, Scotland). For production of the commercial cultures, cryovials of *L. reuteri* NCIMB 30242 are obtained from the Master repository at NCIMB, and production of several working lots are then produced by Chr Hansen. Starter cultures for production of the commercial fermentation batches are then produced using these working batches. Through the application of master and working batches, genomic stability of the organism is therefore assured. Verification of the identity of NCIMB 30242 occurs prior to production of the working cultures and prior to production of a commercial batch (see Figure II.E-1) *via* the use of API 50 CHL and APIzym test strips.

Production of the commercial fermentation batches is conducted under cGMP, using food grade materials[4] that are permitted and suitable for use in the production of food ingredients. Prior to addition of the starter cultures the fermentation media is sterilized, and then cooled to ambient working temperatures suitable for growth of *L. reuteri*. The fermentation media is then inoculated with the NCIMB 30242 starter culture and allowed to incubate to a defined fermentation endpoint under controlled conditions. Following incubation, the pH is adjusted, and the biomass is concentrated using centrifugation to produce a concentrated slurry to which permitted food grade cryoprotectants are added. The slurry is then frozen and freeze dried. The dried powder is then packaged using approved materials and stored under cold dry conditions. The final product then undergoes QC testing for assurance of purity, and absence of heavy metal and microbiological contamination.

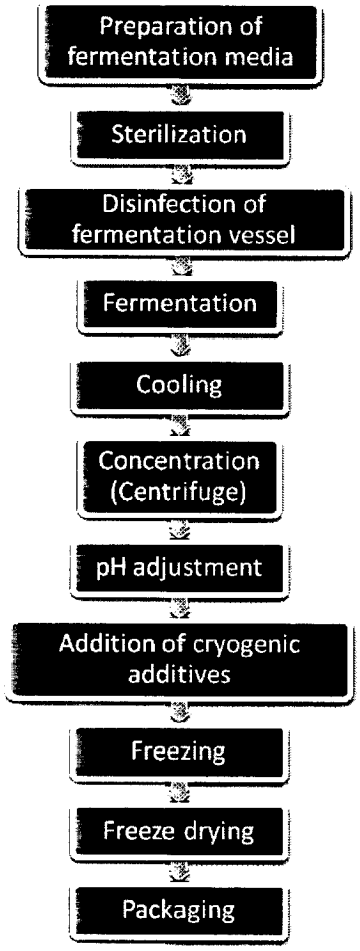

Figure II.E-1 Manufacture of *Lactobacillus reuteri* NCIMB 30242 freeze dried powder

[4] See Appendix B for regulatory compliance letters from CHR Hansen.

(ii) Reformulation

Lactobacillus reuteri are strict anaerobes and therefore are highly susceptible to oxidative environments. To improve the stability/viability of the organism under certain food uses, the lyophilized preparation may be encapsulated/coated with various food grade ingredients. Currently re-formulation of the ingredient is conducted at BRACE GmbH (Karlstein, Germany) under controlled conditions using food grade materials permitted for use in food in the U.S. A general outline of the process is described below. Prior to production, all vessels are sterilized, and the NCIMB 30242 lyophilized powder/frozen slurry is thawed in a water bath at 37°C. The material is then transferred to a solution of NaCl, and sieved through a micron mesh. Sodium alginate, and sodium chloride is added and the solution is raised to neutral pH. The slurry is processed through a nozzle plate with a gelling solution containing calcium chloride and polyethylene glycol (MW = 1500) to produce gel beads, which then proceed through a sequence of washing (sodium chloride) and incubation steps with sodium alginate and epsilon-polylysine to produce a stable food grade coating on the gel particles. The materials used during the encapsulation process and their corresponding regulatory status are presented below in Table II.E-1.

Table II.E-1 Raw Materials Used During Encapsulation and Regulatory Status for Use in the United States

Material	Regulatory Status
Sodium chloride	GRAS – Traditional food ingredient
Calcium chloride	Under 21 CFR §184.1193 calcium chloride is GRAS affirmed for use as a stabilizer and thickener in foods at levels not to exceed cGMP (U.S. FDA, 2011a).
Sodium hydroxide	Under 21 CFR §184.1763 sodium hydroxide is GRAS affirmed for use as a pH control agent limited to cGMP (U.S. FDA, 2011a).
Polyethylene glycol (MW = 1500 Da)	Under 21 CFR §172.820 polyethylene glycol (MW 200 to 9,500 Da) is permitted for use as a coating, binder, plasticizing agent, and/or lubricant in tablets used for food at a use level not to exceed that necessary to achieve desired effect (U.S. FDA, 2011a).
Sodium alginate	Under 21 CFR §184.1724, sodium alginate is GRAS affirmed for general food use as a stabilizer and thickener at a use level of up to 1.0% (U.S. FDA, 2011a).
Epsilon-Polylysine	GRAS for use on cooked rice products at use level up to 0.005% GRAS for use in a multiple food categories, including meat and poultry at use levels between 0.005 to 0.06%

cGMP = current Good Manufacturing Practices; GRAS = Generally Recognized as Safe

(iii) Specifications

A number of ingredient formats are proposed for various food uses of *L. reuteri* NCIMB 30242, and include a frozen slurry, lyophilized powder, and encapsulated powder. Use of a particular format will be dependent upon the particular food application of the material. For example, the

frozen slurry may be used for applications where *L. reuteri* is incorporated in fermentation applications such as yogurt; stringent microbial specifications are therefore applied to this material. Food grade specifications for the frozen slurry, lyophilized powder, and encapsulated material are presented below in Table II.E-2 through II.E-4. Each lot of *L. reuteri* NCIMB 30242 is analyzed for purity and extensive analyses for microbial contamination are conducted following both the fermentation and the encapsulation processes. Heavy metal testing for lead also is conducted on each lot of material.

Table II.E-2 Product Specification for *Lactobacillus reuteri* NCIMB 30242 Frozen Slurry

Parameter	Specification
L. reuteri (API 50 and APIzym strip analyses)	Pass
Enterobacteiaceae	<1 CFU/g
Non lactic acid bacteria	<1 CFU/g
Enterococci	<1 CFU/g
Staphylococci	<1 CFU/g
Staphylococcus aureus	<1 CFU/g
Yeast and moulds	Absent in 25 g
Listeria monocytogenes	Absent in 25 g
Salmonella	Absent in 25 g
Coliforms	<1 CFU/g
Total *Bacillus*	<1 CFU/g
Bacillus cereus	<1 CFU/g
Mesophilic lactic acid bacteria	<1 CFU/g
Streptococcus thermophilus	<1 CFU/g
Mesophilic lactic acid bacteria after subculture	<1 CFU/g
Streptococcus thermophilus after subculture	<1 CFU/g
Non lactic acid bacteria after subculture	<1 CFU/g

CFU = colony forming units
Note: As stated by Chr Hansen, milk based ingredients are used during fermentation of NCIMB 30242, therefore, in accordance with the Food Allergy Labeling and Consumer Protection Act (FALCPA) product labeling of NCIMB 30242 containing products should include "contains milk" in the ingredient list.

Table II.E-3 Product Specification for *Lactobacillus reuteri* NCIMB 30242 Freeze Dried Powder

Parameter	Specification
Non lactic acid bacteria	<100 CFU/g
Escherichia coli	Absent/g
Yeasts	<10 CFU/g
Moulds	<10 CFU/g
Enterococcus spp.	<100 CFU/g
Coagulase positive staphylococcus	<10 CFU/g
Sulfite-reducing bacteria	<10 CFU/g
Salmonella spp.	Absent/25g
Listeria monocytogenes	Absent/g

CFU = colony forming units

Table II.E-4 Product Specification for *Lactobacillus reuteri* NCIMB 30242 Encapsulated Material

Description		
Water content = 80-86% *Lactobacillus reuteri* NCIMB 30242 = 11-15% Alginic acid = 2-3% ε-Polylysine = 0.08% PEG – 1500 (%) = 1% Particle size = 500 µm ± 100 µm Contains milk protein in trace quantities		
Phenotypic and Genotypic Identification		
Parameter	Specification	Method
Cell morphology	Rod (1x2-4 µm); single, pairs, non-motile, no spores	As performed by Belgian Co-ordinated Collection of Microorganisms internal SOP
Gram stain	Positive	
Oxidase reaction	Negative	
Catalase reaction	Negative	
Lactobacillus reuteri (API 50 and APIzym strip analyses)	Pass	
DNA Fingerprinting (AFLP)	≥80% AFLP pair wise profile similarity	
Microbial Analyses		
Lactobacillus reuteri NCIMB 30242	NLT $1x10^8$ CFU/g	NNFA Probiotic
Sulphite reducing anaerobic sporulating bacteria	<1 CFU/3mL	ISO 15213 (modified)
Bacillus cereus	<1 CFU/mL	AFNOR NF ISO 7932
Enterobacteriaceae	<100 CFU/mL	NF EN ISO 21528-1
Enterococcus	<1 CFU/mL	NF EN ISO 11290-1
Listeria monocytogenes	Absent in 25 g	NF ISO 6579
Salmonella	Absent in 25 g	NF EN ISO 6888-2

Table II.E-4 Product Specification for *Lactobacillus reuteri* NCIMB 30242 Encapsulated Material

Staphylococcus aureus (CFU/mL)	<1 CFU/mL	NF EN ISO 6888-2
Yeasts	< 100 CFU/20 mL	ISO 6611-1992
Moulds	< 100 CFU/20 mL	ISO 6611-1992
Metals		
Arsenic	<0.05 ppm	ICP-MS/AOAC 993.14
Cadmium	<0.05 ppm	
Lead	<0.05 ppm	
Mercury	<0.05 ppm	

CFU = colony forming units; NLT = not less than

Batch analyses for 3 non-consecutive lots of encapsulated product are presented in Table II.E-5 below demonstrating that the product is manufactured in a consistent manner in compliance with the product specifications.

Table II.E-5 Batch analyses of *Lactobacillus reuteri* NCIMB 30242 – Encapsulated Product

Parameter	**Specification**	**Lot Number**		
		1430-6010/ 201000164-1	**1430-6010/ 201000114-8**	**1430-6010/ 201000164-2**
Microbial Analyses				
Lactobacillus reuteri NCIMB 30242 (CFU/g)	NLT $2x10^8$	$2.83x10^8$	$1.32x10^8$	$2.52 x10^8$
Sulphite reducing anaerobic sporulating bacteria (CFU/3 mL)	<1	<1	<1	<1
Bacillus cereus (CFU/mL)	<1	<1	Absent	<1
Enterobacteriaceae (CFU/mL)	<100	4.3	<0.3	<0.3
Enterococcus (CFU/mL)	<1	<1	<1	<1
Listeria monocytogenes (CFU/25 g)	ND	ND	ND	ND
Salmonella (CFU/25 g)	ND	ND	ND	ND
Staphylococcus aureus (CFU/mL)	<1	<1	<1	<1
Yeast (CFU/20 mL)	<100	<1	<1	<1
Moulds (CFU/20 mL)	<100	<1	<1	<1
Heavy Metal Analyses				
Arsenic	<0.05	ND	ND	ND
Cadmium	<0.05	ND	0.002	ND
Lead	<0.05	0.023	ND	0.017
Mercury	<0.05	ND	ND	0.005

CFU = colony forming units; ND = Not Detected

F. Stability of *L. reuteri* NCIMB 30242

The stability of *Lactobacillus reuteri* NCIMB 30242 in lyophilized powder and frozen pellet formats, as produced by CHR Hansen, was evaluated. *Lactobacillus reuteri* NCIMB 30242 lyophilized powder was stored refrigerated (2-8°C) with uncontrolled humidity for 12 months. Stability was measured at baseline (Day 0) and after 1, 3, 6, 9 and 12 months. *Lactobacillus reuteri* NCIMB 30242 frozen pellets were stored at -80°C for 13 months. Stability was measured at baseline (Day 0) and after 13 months of storage for Batches 1 and 2 and at baseline (Day 0) and after 8 months of storage for Batches 3, 4 and 5. At each time-point, a representative sample of lyophilized powder or frozen pellet was weighed, brought to room temperature, diluted in sodium chloride casein peptone solution and transferred into a sterile stomacher bag for rehydration using a Stomacher 80 Biomaster Lab Blender. Samples were serial diluted and plated on de Man-Rogosa-Sharpe cysteine agar plates. Plates were incubated in an anaerobic chamber at 37°C for 24 to 48 hours. Viability was calculated as colony forming units per gram (CFU/g) of lyophilized powder or frozen pellet.

As shown in Figure II.F-1, viability of *L. reuteri* NCIMB 30242 as a lyophilized powder was retained after 12 months of refrigerated storage. At baseline and after 12 months, the viability of *Lactobacillus reuteri* NCIMB 30242 was 9.32×10^{10} CFU/g and 6.33×10^{10} CFU/g, respectively. As shown in Figure II.F-2, cell viability of *Lactobacillus reuteri* NCIMB 30242 within a frozen pellet format was retained at 100% for Batches 1 and 2 after 13 months of frozen storage. Furthermore, cell viability of *Lactobacillus reuteri* NCIMB 30242 frozen pellets was retained at 100%, 89% and 100% for Batches 3, 4 and 5 after 8 months of frozen storage.

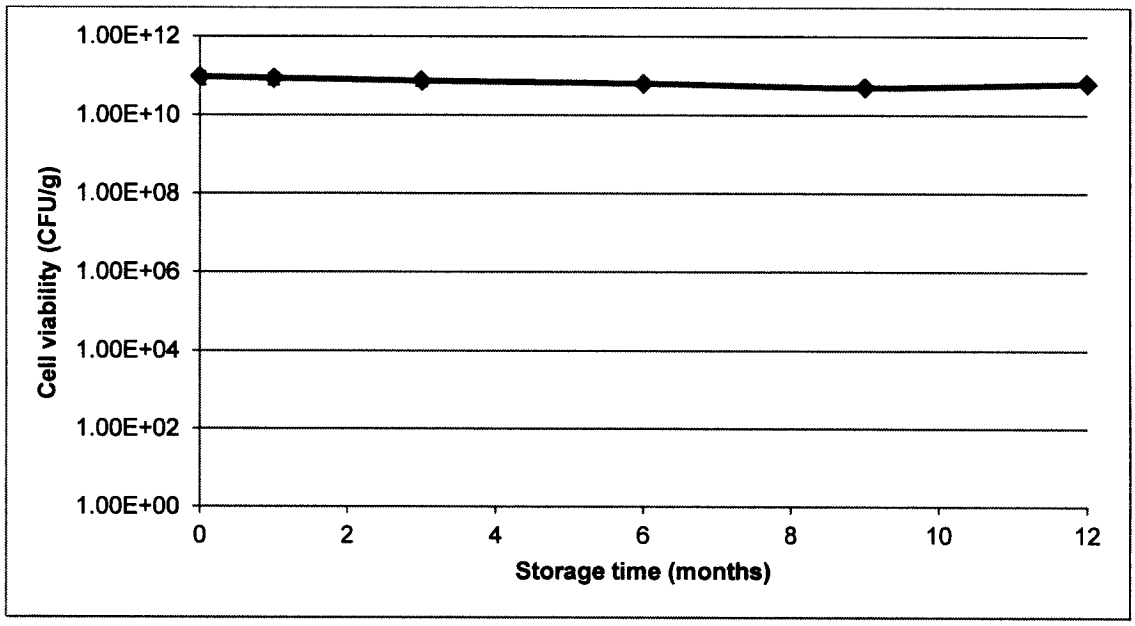

Figure II.F-1 Cell viability, in CFU per gram of *Lactobacillus reuteri* NCIMB 30242 lyophilized powder (CHR Hansen) after 12 months of refrigerated storage (2-8°C) with uncontrolled humidity.

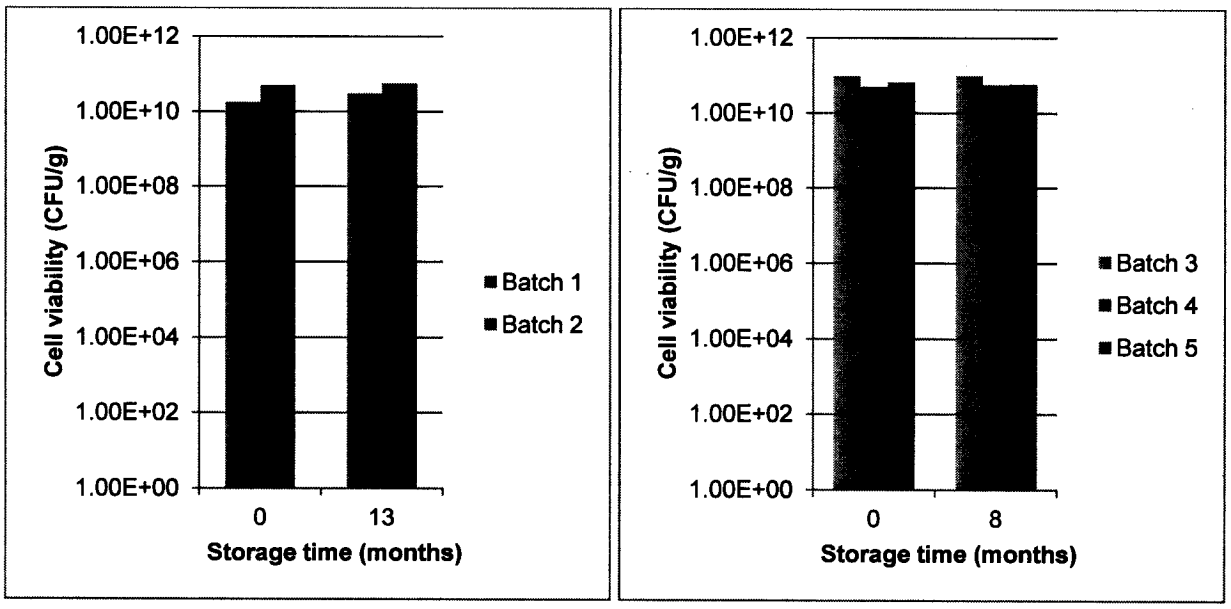

Figure II.F-2 Cell viability, in CFU per gram of *Lactobacillus reuteri* NCIMB 30242 frozen pellet (CHR Hansen) after 13 months (Batches 1 and 2) or 8 months (Batches 3, 4 and 5) of frozen storage (-80°C)

III. SELF-LIMITING LEVELS OF USE

Under the intended conditions of use of *L. reuteri* NCIMB 30242, no self-limiting use levels are expected.

IV. BASIS FOR GRAS DETERMINATION

The data and information summarized below demonstrate that *L. reuteri* NCIMB 30242, under the conditions of proposed use described in Table I.D-1, is GRAS, based on scientific procedures. The use of *L. reuteri* in food has a long-history of safe consumption, and in addition to its common traditional food use in fermented foods, a large body of published clinical and animal studies evaluating the consumption of various strains of *L. reuteri* was identified within the genrally available literature. Available studies evaluating the consumption of *L. reuteri* by humans have been conducted across a broad spectrum of population types including healthy and unhealthy subjects, immunocompromised individuals, and infants and children. These studies are described in Section IV.D, and provide important information characterizing the pathogenicity and toxicogenicity potential of the species; corresponding conclusions in this regard are directly relevant to the safety of *L. reuteri* NCIMB 30242. Additional information relevant to the assessment of pathogenicity of the species *L. reuteri* is present in Section E., and the relevance of available animal data to the safety assessment of *L. reuteri* NCIMB 30242 is discussed (Section IV.C). Published information characterizing the metabolic characteristics, antibiotic resistance, and information obtained from bioinformatic assessment of *L. reuteri* NCIMB 30242 is presented (Sections IV.F – IV.H). Finally, *L. reuteri* NCIMB 30242 exhibits a high capacity for the metabolism of bile acids, a unique phenotypic property that differentiates *L. reuteri* NCIMB 30242 from non-related *L. reuteri* strains. This phenotype has been shown to confer nutritional benefits on lipid metabolism in frequent consumers of foods containing the organism, and a nutritional assessment of this phenotype is discussed (Section IV.I).

Moreover, this data and information were reviewed by a Panel of Experts, qualified by scientific training and experience to evaluate the safety of *L. reuteri* NCIMB 30242 as a food ingredient, who concluded that of *L. reuteri* NCIMB 30242 was GRAS under the aforementioned conditions of intended use in food based on scientific procedures. A summary of the data reviewed by the Expert Panel is presented herein.

A. Intake Estimation

(i) Current Uses and Background Exposure to *L. reuteri* in the Diet

As discussed, *Lactobacillus reuteri* has a long history of apparent safe use by the food industry as a fermentation starter in the manufacture of sourdough and other artisanal breads. However, since most *L. reuteri* strains are strict anaerobes, food exposure to *L. reuteri* strains used in the

manufacture of bread would be limited as the organism would be unlikely to survive the high oxidative environment of the bread baking process.

No federal regulations pertaining to the use of *L. reuteri* in food were identified; however, as per FDA's partial list of microorganisms and microbial derived ingredients used in foods (U.S. FDA, 2001), prior sanctions have been granted for the "use of harmless lactic acid producing bacteria, such as *Lactobacillus acidophilus*", as optional ingredients in specified standardized foods, which include buttermilk, sour cream, cottage cheese, and yogurt. Although no explicit provisions for the addition of lactic acid-producing bacteria are included in the regulatory standards for these products, in January 2009, a proposed regulation to revoke the standards for low-fat yogurt and non-fat yogurt and to amend the standard for yogurt was published in the Federal Register (74FR2443) (U.S. FDA, 2009). Under the proposed ruling, an explicit provision permitting the addition of safe and suitable cultures, in addition to the required characterizing bacterial cultures, was proposed. Thus, the addition of "safe and suitable" lactic acid bacterial cultures, including *L. reuteri*, would be permitted for addition to buttermilk, sour cream, cottage cheese, and yogurt without pre-market Notification.

Non sanctioned food uses of *L. reuteri* require GRAS self-affirmation. To date, one GRAS self-affirmation has been Notified to FDA. As described within GRN 254, *L. reuteri* DSM 17938 is considered GRAS for use in processed cheeses, yogurt, ice cream, fruit juices, fruit drinks, processed vegetables, processed vegetable drinks, beverage bases, energy bars, energy drinks, and chewing gum at a level up to $1x10^9$ colony forming units (CFU) per serving, and in a drinking straw at a level of $1x10^9$ CFU per straw (U.S. FDA, 2008). Cumulative intakes under these proposed uses were estimated to be between $1x10^9$ to $1x10^{10}$ CFU/day.

(ii) Estimated Intake of *L. reuteri* NCIMB 30242

The individual proposed food-uses and use-levels for *Lactobacillus reuteri* NCIMB 30242 employed in the current intake analysis are summarized in Table IV.A-1. Food codes representative of each proposed food-use were chosen from the National Health and Nutrition Examination Survey (NHANES) 2003-2006 (CDC, 2006, 2009; USDA, 2009). Food codes were grouped in food-use categories according to Title 21, Section §170.3 of the Code of Federal Regulations (U.S. FDA, 2011a). Product-specific adjustment factors were developed based on data provided in the standard recipe file for the Continuing Survey of Food Intakes by Individuals (CSFII) 1994-1996, 1998 survey (USDA, 2000).

Table IV.A-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *Lactobacillus reuteri* NCIMB 30242 in the United States (2003-2006 NHANES Data)

Food Category	Proposed Food-Uses	*L. reuteri* Use Level (g/serving)	Serving Size (g or mL)[c]	*L. reuteri* Use-Level (%)[b]
Beverages and Beverage Bases	Meal Replacement Beverages	$1x10^{10}$	240	0.17
Breakfast Cereals	Ready-to-Eat Breakfast Cereals	$1x10^{10}$	15 (Puffed)	2.67
			30 (Regular)	1.33
			55 (Biscuit-Type)	0.73
Cheeses	Cream Cheese[c]	$3.3x10^{8}$	30	0.43
	Natural Cheese[c]	$3.3x10^{8}$	30	0.43
	Processed Cheese and Spreads[c]	$3.3x10^{8}$	30	0.43
Dairy Product Analogs	Soy-Based Beverages	$1x10^{10}$	240	0.17
Fats and Oils	Butter	$3.3x10^{8}$	15	0.87
	Fat-Based Sauces	$3.3x10^{8}$	30	0.43
	Margarine[c] and Margarine-like Spreads	$3.3x10^{8}$	15	0.87
	Mayonnaise[c] and Mayonnaise-Type Dressings	$3.3x10^{8}$	15	0.87
	Salad Dressings[c]	$3.3x10^{8}$	30	0.43
	Vegetable Oils	$3.3x10^{8}$	15	0.87
Frozen Dairy Desserts	Frozen Novelties and Frozen Milk Desserts	$1x10^{10}$	120	0.33
	Frozen Yogurt	$1x10^{10}$	120	0.33
	Ice Cream[c]	$1x10^{10}$	120	0.33
			240 (sundaes)	0.17
Grain Products and Pastas	Cereal and Granola Bars	$1x10^{10}$	40	1.00
	Energy, Meal Replacement, and Fortified Bars	$1x10^{10}$	40	1.00
Milk Products	Fermented Milks (plain)[c]	$1x10^{10}$	240	0.17
	Flavored Milk, Milk Drinks, and Mixes	$1x10^{10}$	120 (eggnog)	0.33
			240	0.17
	Milk-Based Meal Replacement Beverages	$1x10^{10}$	240	0.17
	Sour Cream[c]	$3.3x10^{8}$	30	0.43
	Yogurt[c]	$1x10^{10}$	225	0.18
	Yogurt Drinks[d]	$1x10^{10}$	240	0.17
Processed Fruits and Fruit Juices	Fruit Juices	$1x10^{10}$	240	0.17

Table IV.A-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *Lactobacillus reuteri* NCIMB 30242 in the United States (2003-2006 NHANES Data)

Food Category	Proposed Food-Uses	*L. reuteri* Use Level (g/serving)	Serving Size (g or mL)[c]	*L. reuteri* Use-Level (%)[b]
Soft Candy	Chocolate Confectionary	$3.3x10^8$	40	0.33
Sugar Substitutes	Sugar Substitutes	$3.3x10^8$	4	3.25

[a] Serving sizes were based on Reference Amounts Customarily Consumed (RACC) per Eating Occasion in the U.S. CFR (21 CFR §101.12) (U.S. FDA, 2011a).
[b] Based on estimate that 400 mg of lyophilized *L. reuteri* powder contains $1x10^{10}$ CFU
[c] These food-uses represent non-standardized food products; however, in order to obtain a conservative intake estimate, surrogate codes for the standardized food products were chosen.
[d] No food codes were identified for yogurt drinks; therefore, surrogate codes of fruit smoothie drinks were used to represent the food codes in this category.

The estimated total intake of *L. reuteri* NCIMB 30242 per mg/person/day and per CFU/person/day from proposed food-uses in the U.S. by population group is summarized in Table IV.A-2. Table IV.B-3 presents these data on a per kilogram body weight basis.

Approximately 93.5% of the total U.S. population were identified as potential consumers of *L. reuteri* NCIMB 30242 from proposed food-uses (15,607 actual users identified). A high percentage of users were identified in each of the individual population groups (94.9 to 99.0%), with the exception of the infant population group (69.1% users). As a result of the high percentage of users identified within all population groups, except for the infant population group, the intake estimates for the all-person and all-user categories were similar; therefore, only the all-user results are discussed in detail. Consumption of proposed food-uses by the total U.S. population resulted in an estimated mean all-user intake of *L. reuteri* NCIMB 30242 of $1.8x10^{10}$ CFU/person/day, which is equivalent to $0.04x10^{10}$ CFU/kg body weight/day. The 90th percentile all-user intake of *L. reuteri* NCIMB 30242 from proposed food-uses by the total population was $3.5x10^{10}$ CFU/person/day, which corresponds to $0.08x10^{10}$ CFU/kg body weight/day.

On an individual population basis, the greatest mean all-user intake of *L. reuteri* NCIMB 30242 on an absolute basis was determined to occur in children and male teenagers at $2.3x10^{10}$ CFU/person/day. Infants and female adults displayed the lowest mean all-user intake of *L. reuteri* NCIMB 30242 on an absolute basis with a value of $1.5x10^{10}$ CFU/person/day. On a body weight basis, the mean all-user intake of *L. reuteri* NCIMB 30242 was highest in infants with an intake of $0.13x10^{10}$ CFU/kg body weight/day. The lowest all-user mean intake on a per kilogram body weight basis was observed to occur in female and male adults with a value of $0.02x10^{10}$ CFU/kg body weight/day.

Table IV.A-2 Summary of the Estimated Daily Intake of *Lactobacillus reuteri* NCIMB 30242 per CFU from Proposed Food-Uses in the U.S. by Population Group (2003-2006 NHANES Data)

Population Group	Age (years)	Percent Users	Actual # of Users	All Person (CFU)		All User (CFU)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 2	69.1	1,322	$1.3x10^{10}$	$3.0x10^{10}$	$1.5x10^{10}$	$3.3x10^{10}$
Children	3 to 11	99.0	2,707	$2.3x10^{10}$	$3.8x10^{10}$	$2.3x10^{10}$	$3.8x10^{10}$
Female Teenagers	12 to 19	96.8	1,924	$1.8x10^{10}$	$3.3x10^{10}$	$1.8x10^{10}$	$3.3x10^{10}$
Male Teenagers	12 to 19	94.9	1,841	$2.0x10^{10}$	$4.3x10^{10}$	$2.3x10^{10}$	$4.3x10^{10}$
Female Adults	20 and up	97.3	4,167	$1.5x10^{10}$	$3.0x10^{10}$	$1.5x10^{10}$	$3.3x10^{10}$
Male Adults	20 and up	94.9	3,646	$1.8x10^{10}$	$3.8x10^{10}$	$1.8x10^{10}$	$3.8x10^{10}$
Total Population	All Ages	93.5	15,607	$1.8x10^{10}$	$3.5x10^{10}$	$1.8x10^{10}$	$3.5x10^{10}$

CFU = colony forming unit

When heavy consumers (90th percentile) were assessed, the estimate for the all-user intake of *L. reuteri* NCIMB 30242 from proposed food-uses was determined to be greatest in male teenagers at $4.3x10^{10}$ CFU/person/day. The lowest 90th percentile all-user intake estimate was observed to occur in infants, female teenagers, and female adults, each with a value of $3.3x10^{10}$ CFU/person/day, on an absolute basis. On a body weight basis, infants were determined to have the greatest all-user 90th percentile intake of *L. reuteri* NCIMB 30242 with a value of $0.26x10^{10}$ CFU/kg body weight/day. The lowest all-user 90th percentile intake of *L. reuteri* NCIMB 30242 on a body weight basis was observed to occur in female and male adults at $0.05x10^{10}$ CFU/kg body weight/day.

Table IV.A-3 Summary of the Estimated Daily Per Kilogram Body Weight Intake of *Lactobacillus reuteri* NCIMB 30242 per CFU from Proposed Food-Uses in the U.S. by Population Group (2003-2006 NHANES Data)

Population Group	Age (years)	Percent Users	Actual # of Users	All Person (CFU/kg bw)		All User (CFU/kg bw)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 2	69.1	1,322	$0.10x10^{10}$	$0.25x10^{10}$	$0.13x10^{10}$	$0.26x10^{10}$
Children	3 to 11	99.0	2,707	$0.09x10^{10}$	$0.17x10^{10}$	$0.09x10^{10}$	$0.17x10^{10}$
Female Teenagers	12 to 19	96.8	1,924	$0.03x10^{10}$	$0.07x10^{10}$	$0.03x10^{10}$	$0.07x10^{10}$
Male Teenagers	12 to 19	94.9	1,841	$0.03x10^{10}$	$0.07x10^{10}$	$0.04x10^{10}$	$0.07x10^{10}$
Female Adults	20 and up	97.3	4,167	$0.02x10^{10}$	$0.05x10^{10}$	$0.02x10^{10}$	$0.05x10^{10}$
Male Adults	20 and up	94.9	3,646	$0.02x10^{10}$	$0.05x10^{10}$	$0.02x10^{10}$	$0.05x10^{10}$
Total Population	All Ages	93.5	15,607	$0.03x10^{10}$	$0.08x10^{10}$	$0.04x10^{10}$	$0.08x10^{10}$

bw = body weight; CFU = colony forming units

(iii) Estimated Consumption of Microencapsulation Additives from the Proposed Food Uses

The *L. reuteri* NCIMB 30242 lyophilized powder may be reformulated/encapsulated with food grade ingredient to improve stability of the organism. Food additives (sodium alginate, polyethylene glycol, and ε-polylysine) currently approved/permitted for use in food in the United States are used for the coating/encapsulation process. The reformulated product is expected to weight between 1 to 2 g per $1x10^{10}$ CFU quantity of *L. reuteri* NCIMB 30242, with approximately 80% of the material being accounted for by water. Based on the production process, sodium alginate, polyethylene glycol and ε-polylysine will be present with the ingredient at maximum concentrations of 30, 10, and 0.5 mg/g of material respectively. These concentrations are considered overestimates, and the actual levels are likely to be lower in the commercial product. Based on these concentrations, estimated intakes of these additives was determined, and are provided in Table IV.A-4 through IV.A-9 below.

Table IV.A-4 Summary of the Estimated Daily Intake of Sodium Alginate from All Proposed Food-Uses of *Lactobacillus reuteri* NCIMB 30242 in the U.S. by Population Group (2003-2006 NHANES Data)

Population Group	Age (years)	Percent Users	Actual # of Users	All Person (mg)		All User (mg)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 2	69.1	1,322	109	268	139	294
Children	3 to 11	99.0	2,707	193	345	194	345
Female Teenagers	12 to 19	96.8	1,924	153	296	157	298
Male Teenagers	12 to 19	94.9	1,841	184	378	192	387
Female Adults	20 and up	97.3	4,167	138	281	141	284
Male Adults	20 and up	94.9	3,646	157	327	163	332
Total Population	All Ages	93.5	15,607	153	315	159	318

Table IV.A-5 Summary of the Estimated Daily Per Kilogram Body Weight Intake of Sodium Alginate from All Proposed Food-Uses of *Lactobacillus reuteri* NCIMB 30242 in the U.S. by Population Group (2003-2006 NHANES Data)

Population Group	Age (years)	Percent Users	Actual # of Users	All Person (mg/kg bw)		All User (mg/kg bw)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 2	69.1	1,322	9	22	11	24
Children	3 to 11	99.0	2,707	8	15	8	15
Female Teenagers	12 to 19	96.8	1,924	3	6	3	6
Male Teenagers	12 to 19	94.9	1,841	3	6	3	7
Female Adults	20 and up	97.3	4,167	2	4	2	4
Male Adults	20 and up	94.9	3,646	2	4	2	4
Total Population	All Ages	93.5	15,607	3	7	3	7

Table IV.A-6 Summary of the Estimated Daily Intake of Polyethylene Glycol from All Proposed Food-Uses of *Lactobacillus reuteri* NCIMB 30242 in the U.S. by Population Group (2003-2006 NHANES Data)

Population Group	Age (years)	Percent Users	Actual # of Users	All Person (mg)		All User (mg)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 2	69.1	1,322	36	89	46	98
Children	3 to 11	99.0	2,707	64	115	65	115
Female Teenagers	12 to 19	96.8	1,924	51	99	52	99
Male Teenagers	12 to 19	94.9	1,841	61	126	64	129
Female Adults	20 and up	97.3	4,167	46	94	47	95
Male Adults	20 and up	94.9	3,646	52	109	54	111
Total Population	All Ages	93.5	15,607	51	105	53	106

Table IV.A-7 Summary of the Estimated Daily Per Kilogram Body Weight Intake of Polyethylene Glycol from All Proposed Food-Uses of *Lactobacillus reuteri* NCIMB 30242 in the U.S. by Population Group (2003-2006 NHANES Data)

Population Group	Age (years)	Percent Users	Actual # of Users	All Person (mg/kg bw)		All User (mg/kg bw)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 2	69.1	1,322	3.0	7.4	3.8	7.9
Children	3 to 11	99.0	2,707	2.6	4.9	2.6	4.9
Female Teenagers	12 to 19	96.8	1,924	0.9	1.9	0.9	1.9
Male Teenagers	12 to 19	94.9	1,841	1.0	2.2	1.0	2.2
Female Adults	20 and up	97.3	4,167	0.6	1.4	0.7	1.4
Male Adults	20 and up	94.9	3,646	0.6	1.3	0.6	1.4
Total Population	All Ages	93.5	15,607	1.0	2.3	1.0	2.3

Table IV.A-8 Summary of the Estimated Daily Intake of ε-Polylysine from All Proposed Food-Uses of *Lactobacillus Reuteri* NCIMB 30242 in the U.S. by Population Group (2003-2006 NHANES Data)

Population Group	Age (years)	Percent Users	Actual # of Users	All Person (mg)		All User (mg)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 2	69.1	1,322	2	4	2	5
Children	3 to 11	99.0	2,707	3	6	3	6
Female Teenagers	12 to 19	96.8	1,924	3	5	3	5
Male Teenagers	12 to 19	94.9	1,841	3	6	3	6
Female Adults	20 and up	97.3	4,167	2	5	2	5
Male Adults	20 and up	94.9	3,646	3	5	3	6
Total Population	All Ages	93.5	15,607	3	5	3	5

Table IV.A-9 Summary of the Estimated Daily Per Kilogram Body Weight Intake of ε-Polylysine from All Proposed Food-Uses of *Lactobacillus reuteri* NCIMB 30242 in the U.S. by Population Group (2003-2006 NHANES Data)

Population Group	Age (years)	Percent Users	Actual # of Users	All Person (mg/kg bw)		All User (mg/kg bw)	
				Mean	90th Percentile	Mean	90th Percentile
Infants	0 to 2	69.1	1,322	0.15	0.37	0.19	0.39
Children	3 to 11	99.0	2,707	0.13	0.25	0.13	0.25
Female Teenagers	12 to 19	96.8	1,924	0.04	0.10	0.05	0.10
Male Teenagers	12 to 19	94.9	1,841	0.05	0.11	0.05	0.11
Female Adults	20 and up	97.3	4,167	0.03	0.07	0.03	0.07
Male Adults	20 and up	94.9	3,646	0.03	0.07	0.03	0.07
Total Population	All Ages	93.5	15,607	0.05	0.11	0.05	0.12

In the United States sodium alginate is GRAS affirmed for general food use as a stabilizer and thickener at a use level of up to 1.0% (U.S. FDA, 2011a). The use (stabilizer, thickener, gelling agent, and emulsifier) of sodium alginate was evaluated by the Joint Expert Committee on Food Additives (JECFA), and an acceptable daily intake (ADI) of 'not specified' was established during their 39th meeting (JECFA, 1992). Polyethylene glycols (MW = 200 to 9,500 Da) are permitted for use in the United States under 21 CFR 172.820 as coating, binding, plasticizing agents, and/or lubricants in tablets at a use level not to exceed that necessary to achieve the desired effect (U.S. FDA, 2011a). An ADI of 0 to 10 mg/kg body weight was established for the use of polyethylene glycols as a carrier solvent/excipient during the committee's 23rd meeting (JECFA, 1979). Finally, ε-polylysine is GRAS self-affirmed for use in multiple food categories at use levels of up to 0.03%; estimated exposures of 8.4 mg/kg body weight were determined to be GRAS (GRN 336 – U.S. FDA, 2011b).

(iv) Summary

Consumption data and information pertaining to the individual proposed food-uses of *Lactobacillus reuteri* NCIMB 30242 were used to estimate the all-person and all-user intakes of *Lactobacillus reuteri* NCIMB 30242 for specific demographic groups and for the total U.S. population. This type of intake methodology is generally considered to be 'worst case' as a result of several conservative assumptions made in the consumption estimates. For example, it is often assumed that all food products within a food category contain the ingredient at the maximum specified level of use. In addition, it is well established that the length of a dietary survey affects the estimated consumption of individual users. Short-term surveys, such as the typical 2- or 3-day dietary surveys, overestimate the consumption of food products that are consumed relatively infrequently.

In summary, on an all-user basis, the mean intake of *L. reuteri* NCIMB 30242 by the total U.S. population from proposed food-uses was estimated to be 1.8×10^{10} CFU/person/day, which is equivalent to 0.04×10^{10} CFU/kg body weight/day. The heavy consumer (90th percentile) all-user intake of *Lactobacillus reuteri* NCIMB 30242 by the total U.S. population from proposed food-uses of *L. reuteri* NCIMB 30242 was estimated to be 3.5×10^{10} CFU/person/day, which corresponds to 0.08×10^{10} CFU/kg body weight/day.

On an individual population basis, the greatest mean all-user intake of *L. reuteri* NCIMB 30242 was determined to occur in children and male teenagers. *L. reuteri* NCIMB 30242 will be intended for sale in premium priced products intended for consumers seeking cardiovascular health type products, thus exposures in children and teenagers will be limited and the calculated intakes in these population groups is not expected to be well represented by the intake estimates. Estimated exposure in 90th percentile female and male all-users was 3.3×10^{10} and 3.8×10^{10} CFU/person respectively.

Since many of the pivotal studies characterizing the safety of a microorganism for food use are strain specific, extrapolation of safety data on *L. reuteri* NCIMB 30242 to cumulative dietary exposures from other *L. reuteri* strains available on the market was considered inappropriate. Thus, estimation of cumulative exposure to *L. reuteri* from the proposed food uses of *L. reuteri* NCIMB 30242, and from background exposure to other *L. reuteri* strains permitted for use in food in the United States was not conducted.

Estimated exposure to the reformulation ingredients also was considered, as expected, based on their low level of use as encapsulation/coating agents, and the limited use level of *L. reuteri* NCIMB 30242 in food, the exposures to these materials is expected to be below the background exposure to these compounds from their current regulated use in food in the United States. The introduction of encapsulated *L. reuteri* NCIMB 30242 would therefore not appreciably increase exposure to these materials in the diet and therefore would be considered GRAS.

B. Metabolic Fate

(i) Animal Studies

There is no strain or species specific information on the metabolic fate of *L. reuteri*; however, it is likely that the level of metabolism of the bacteria would be dependent upon the degree to which the organism is able to survive gastrointestinal transit, and bacteria not surviving transit are expected to be used as an energy source (protein, lipids, carbohydrates) in a similar manner to that reviewed for *Lactobacillus johnsonii* La-1 below, and the remaining non-digestible components would be metabolized by resident bacteria within the colon, and/or excreted in the feces.

Wutzke and Oetjens (2005) investigated the metabolic fate of double labeled (dl) ^{13}C-and ^{15}N-*Lactobacillus johnsonii* La-1 in humans. The study was conducted in 10 healthy adults (8 female, 2 male; mean age = 25.9 y; mean body weight = 67.4 kg). During the morning of the study, each subject was administered yoghurt containing dl *L. johnsonii* La-1 (dl-La-1) at a dose of 86.6 mg per kg of body weight (number of microbes administered not reported), and blood, breath, urine, and feces samples were collected periodically over a 2-day period. A prompt increase in $^{13}CO_2$ was observed within 30 minutes of dosing, peaking between 2 to 4 hours. Breath hydrogen levels began to increase between 3 to 4 hours peaking at 5 hours post dl-La-1 consumption. Urinary ammonia peaked at 2 hours and total urinary nitrogen levels gradually increased from t=0, reaching maximum levels within 5 hours. Over the 2-day period 12.4% of the ingested ^{15}N dose was excreted in the urine as urea, the main protein degradation product. The total expired and urine $^{13}CO_2$ excretion was 9.2%, with 86% of the total accounted for by expired breath $^{13}CO_2$. Fecal radioactivity accounted for 40% of the administered dose. The results of this experiment indicate that *L. johnsonii* La-1 is rapidly degraded by intestinal digestion following consumption, with a significant amount of cecal bacteria degradation of presumably killed microbes accounting for a significant proportion of radioactivity by Day 2. The

authors determined that approximately 50% of the ingested dose of dl-La-1 was metabolized to simple innocuous compounds. The number of viable organisms remaining in the colon could not be determined.

Wutzke *et al.* (2008) conducted a subsequent study using the same dl-La-1; however in this experiment the bacteria were heat-killed before consumption. The study used a similar study design to the previous experiment. The heat killing procedure displayed little effect on the radioactivity distribution over the 2 days, and a similar pattern in ^{13}C and ^{15}N excretion to that reported above was observed. Compared to the consumption of live dl-La-1 where 50% of the administered dose was metabolized and excreted, the ingestion of heat-killed bacteria resulted in a loss of 55 to 60% of the administered dose to metabolism. The difference in radioactivity excretion between the 2 experiments suggests that a small amount of live bacteria were able to survive gastrointestinal transit and colonize the gut. Similar to the previous study the products of dl-La-1 metabolism were used by the body as nutrient sources (protein, carbohydrate) and metabolized by the body through readily predictable metabolic pathways.

(ii) Human Studies

No information reporting the metabolism of orally administered *L. reuteri* in humans was identified. Based on the improvements in cholesterol homeostasis observed in hypercholesterolemic subjects consuming the organism on a repeated basis (see Section IV.I), *L. reuteri* NCIMB 30242 is expected to largely survive gastrointestinal transit. Transient survival throughout the small intestine and colon is expected. Microorganisms not surviving gastrointestinal transit are expected to be digested by normal metabolic processes of digestion. The remaining microorganisms not subject to digestive processes would be excreted in the feces.

C. Toxicology Studies

Toxicology studies conducted with *L. reuteri* or related species were not identified in the literature. Microorganism host interactions are species specific. For example, as reported by Johansson *et al.* (1993) the human *L. reuteri* isolate (LR108) was unable to colonize the rat intestine, and following inoculation of several human subjects with the *L. reuteri* rat isolate LR47, a strain demonstrating excellent colonization capacity in the rat, the strain could not be recovered in any of the subjects. Thus, based on the fact that the capacity of a microorganism to thrive, and interact with its host are species specific, observations obtained from studies (*i.e.*, rodent toxicology studies) conducted in animals, rodents in particular, are of limited relevance to humans. This viewpoint was recently emphasized by a Panel of Experts Qualified for evaluation of microbial safety who stated that "for the safety related endpoints important in assessment of probiotics, validated animal models do not exist and, as a result, the determination of safety rests primary on human studies." (Shane *et al.*, 2010).

Thus, based on limitations in the usefulness of rodent studies for the safety assessment of microorganisms intended for human food use, the safety of *L. reuteri* NCIMB 30242 is largely based on the generally recognized long-history of safe consumption of the species *L. reuteri* by humans, and product specific safety information obtained from the conduct of a placebo controlled study in healthy human subjects consuming *L. reuteri* NCIMB 30242 on a repated basis. This information is discussed in section IV.D below.

D. Human Studies

(i) *Lactobacillus reuteri* NCIMB 30242

The safety and tolerance of *L. reuteri* NCIMB 30242 has been evaluated in double-blinded, placebo-controlled, randomized, parallel-arm multi-center study. The study lasted a total of 10 weeks, and included a 2-week wash-out period, a 2-week run-in period in which subjects consumed placebo yogurts twice daily at breakfast or dinner, and a 6-week treatment period in which subjects consumed either placebo or treatment yogurts twice daily at breakfast or dinner. *Lactobacillus reuteri* NCIMB 30242 was fermented in compliance with SOPs and microbiological analyses and culture purity were confirmed immediately after each production batch. These production batches were then reformulated with alginate-polylysine-alginate coating to improve stability of the organism[5] and the final viability of the lyophilized powder was $5x10^9$ CFU/g. Yogurt test articles were produced using standard manufacturing procedures for yogurt production: milk fatness was adjusted, milk homogenized, pasteurized, cooled to fermentation temperature, starter culture was added, and the batch was stirred. Placebo yogurts were filled to a weight of 125 g in plastic cups. For the test yogurts, an adequate weighted ratio of microcapsules were added to the yogurt, stirred and added to plastic cups. The study product contained 115 g of yogurt and 10 g of microcapsules. Yogurt fat and protein content were 1 g/100 g yogurt and 6.3 g/100 g yogurt respectively. The study product was supplemented with 10 g microencapsulated bile salt hydrolase (BSH)-producing *L. reuteri* NCIMB 30242 (approximately $5x10^{10}$ CFU). The yogurts were produced 5 times during the study with the batch numbers 1, 2, 3, 4, and 5. Each batch number was the same for the study yogurt as well as for placebo. The expiry date of both study and placebo yogurts were determined to be 3 weeks after the yogurt production for each batch. Composition of placebo and treatment yogurts is shown in Table IV.D-1.

[5] This coating process was identical to that described in Section II.E, and is considered representative of the commercial product.

Table IV.D-1 Composition of Placebo and Treatment Yogurts

Content per 100g yogurt	Placebo	Treatment
Protein (g)	6.3	6.3
Carbohydrates (g)	9.2	9.2
Lipids (g)	1	1
Yogurt bacteria (CFU/mL)	$1x10^7$	$1x10^7$
microencapsulated *Lactobacillus reuteri* NCIMB 30242 (CFU)[1]	0	$5.0x10^{10}$
microencapsulated *L. reuteri* NCIMB 30242 (CFU)[2]	0	$1.4x10^9$

CFU = Colony Forming Unites
[1] Calculated using microcapsule viability at production (CFU/g microcapsules).
[2] Calculated using on microcapsule viability as measured after international shipping at 4°C (CFU/g microcapsules).

Blood for assessment of lipid profile was collected at each visit. Serum samples were analyzed enzymatically for low-density lipoprotein (LDL)-cholesterol (primary efficacy variable), total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, and ApoB-100. Blood for assessment of safety profile was collected at baseline and endpoint. Whole blood (hematology) was analyzed for hemoglobin, hematocrit, red blood cells, white blood cells, and platelets. Serum (biochemistry) was analyzed for urea, creatinine, bilirubin, SGOT, SGPT, GGT, AlkP, glucose, calcium, phosphate, potassium, sodium, chloride, bicarbonate, and lipase.

Fecal samples were collected in the 3 days before baseline and each endpoint. Fecal microflora count and pattern was assessed using real time polymerase chain reaction (PCR). The following groups of bacteria and fungi were assessed using published and confirmed primer sequences: *Eubacteria*, *Bacteroides/Prevotella*, *Lactobacillus/Streptococcus/ Enterococcus*, *Bifidobacterium*, *Faecalibacterium*, *Desulfovibrio*, *Enterobacteria*, *Clostridium leptum*, *Clostridium coccoides*, *Saccharomyces*, *Candida*, and *Aspergillus*. Fecal bile acid concentration was determined using gas-liquid chromatography as described by Batta *et al.* (2002).

Three hundred-seventy-five (375) patients underwent initial screening, 159 were enrolled, 120 were randomized, 114 completed the study as part of the intention to treat population (ITT) and 109 completed the study as part of the per protocol population (PP). All patients were considered hypercholesterolemic, at borderline, high, or very high risk of heart disease according the National Cholesterol Education Program Guidelines (NCEP) at the study baseline. The baseline characteristics for the 120 subjects randomized into the study (safety population) were similar between treatment and placebo groups and are presented in Table IV.D-2.

Table IV.D-2 Baseline Characteristics of the Subjects

	Placebo	*Lactobacillus reuteri* NCIMB 30242
Male/female	21/40	22/37
Age (years)	48.85 ± 13.17	52.02 ± 13.20
Body weight (kg)	76.16 ± 11.65	75.83 ± 10.93
BMI (kg/m^2)	26.17 ± 2.61	26.14 ± 2.82
Heart rate (bpm)	75.93 ± 8.90	77.00 ± 8.16
Systolic blood pressure (mmHg)	132.28 ± 10.50	135.10 ± 13.01
Diastolic blood pressure (mmHg)	78.20 ± 6.42	79.66 ± 6.84
Fasting blood glucose (mmol/l)	5.08 ± 0.71	5.18 ± 0.91
Statin intake (%)	0	0

BMI – Body Mass Index; bpm = beats per minute

Following the 6-week treatment period, consumption of *L. reuteri* NCIMB 30242 significantly reduced the LDL-C concentration, from baseline as compared to placebo, by 8.92% (p=0.006) and 9.23% (p=0.006; data not shown) for ITT and PP populations respectively. Also over 6 weeks treatment, significant reductions of 4.81% (p=0.045) in total cholesterol (TC), and of 6.81% (p=0.046) in apoB-100, from baseline as compared to placebo, were seen. Finally, a non-significant increase of 0.1% (p=0.982) in HDL-C was seen at the 6-week endpoint. A detailed overview of the effect of *L. reuteri* NCIMB 30242 on measures of cholesterol metabolism is provided in Section IV.I.

Fecal samples were collected at baseline and at the Week 6 endpoint, and were analyzed by qPCR for total DNA, Eubacteria, Bacteroides/Prevotella, *Lactobacillus/Streptococcus/ Enterococcus*, *Bifidobacterium*, *Faecalibacteria*, *Desulfovibrio*, *Enterobacteria*, *Clostridium leptum*, *Clostridium coccoides*, *Saccharomyces*, *Candida*, and *Aspergillus*. Information obtained from fecal microbial analyses showed a significant decrease in *Aspergillus* DNA from baseline, when comparing treatment and placebo groups. All other bacterial and fungal measurements showed no significant changes between treatment and placebo groups from baseline. No significant between group differences in fecal bile acid concentrations were observed at the 6-week time point.

As shown in Table IV.D-3 below, the between group biochemical and hematological parameters were comparable at baseline, and no significant between group differences were noted for any of the safety parameters over the 6-week treatment period. Further, the number of subjects in placebo and treatment groups with clinically significant values (CSV) for biomarkers was determined and totaled 6 subjects in the placebo group and 1 subject in the treatment group.

Table IV.D-3 Biochemical Markers of Safety for Treatment and Placebo Groups at Baseline and Endpoint are Given and Number of Clinically Significant Values (CSV) for Subjects is Recorded

Parameters	Week	Mean ± SD	CSV	Mean ± SD	CSV
WBC [10^9/l]	Week 0	6.69 ± 1.71	0	7.08 ± 1.66	0
	Week 6	6.91 ± 1.85	0	6.90 ± 1.65	0
RBC [10^{12}/l]	Week 0	4.73 ± 0.44	0	4.75 ± 0.37	0
	Week 6	4.72 ± 0.39	0	4.75 ± 0.41	0
Platelets [10^9/l]	Week 0	263.10 ± 44.62	0	266.74 ± 53.76	0
	Week 6	265.45 ± 46.30	0	265.49 ± 55.23	0
Hematocrit [1]	Week 0	0.42 ± 0.04	0	0.41 ± 0.03	0
	Week 6	0.42 ± 0.03	0	0.42 ± 0.03	0
Hemoglobin [g/l]	Week 0	143.27 ± 12.61	0	142.18 ± 9.72	0
	Week 6	142.58 ± 11.52	0	142.44 ± 10.83	0
Urea [mmol/l]	Week 0	5.24 ± 1.82	1	5.31 ± 1.85	0
	Week 6	5.17 ± 1.68	1	4.76 ± 1.37	0
Creatinine [µmol/l]	Week 0	81.78 ± 27.90	1	76.16 ± 18.18	0
	Week 6	83.58 ± 30.32	1	82.56 ± 17.71	0
Bilirubin [µmol/l]	Week 0	9.21 ± 4.16	0	8.79 ± 4.15	0
	Week 6	9.37 ± 3.98	0	8.91 ± 3.74	0
SGPT/ALT [µkat/l]	Week 0	0.72 ± 0.19	0	0.72 ± 0.24	0
	Week 6	0.74 ± 0.21	0	0.75 ± 0.24	0
SGOT/AST [µkat/l]	Week 0	0.39 ± 0.12	0	0.40 ± 0.15	0
	Week 6	0.40 ± 0.14	0	0.40 ± 0.14	0
GGT [µkat/l]	Week 0	0.73 ± 0.67	0	0.58 ± 0.32	0
	Week 6	0.68 ± 0.64	1	0.75 ± 0.61	1
ALP [µkat/l]	Week 0	1.16 ± 0.46	0	1.25 ± 0.46	0
	Week 6	1.46 ± 0.39	1	1.39 ± 0.30	0
Glucose [mmol/l]	Week 0	5.08 ± 0.71	0	5.18 ± 0.91	0
	Week 6	5.20 ± 0.59	0	5.11 ± 0.56	0
Calcium [mmol/l]	Week 0	2.18 ± 0.11	0	2.20 ± 0.10	0
	Week 6	2.20 ± 0.13	1	2.18 ± 0.08	0
Phosphorus [mmol/l]	Week 0	1.06 ± 0.17	0	1.10 ± 0.14	0
	Week 6	1.18 ± 0.20	0	1.11 ± 0.16	0
Sodium [mmol/l]	Week 0	138.52 ± 2.90	0	138.66 ± 2.27	0
	Week 6	137.02 ± 2.75	0	137.80 ± 2.35	0
Potassium [mmol/l]	Week 0	4.30 ± 0.49	0	4.31 ± 0.43	1
	Week 6	4.39 ± 0.45	0	4.38 ± 0.39	0
Chloride [mmol/l]	Week 0	100.10 ± 2.47	0	100.24 ± 2.23	0
	Week 6	99.22 ± 2.23	0	99.85 ± 1.81	0
Bicarbonate [mmol/l]	Week 0	22.25 ± 1.20	0	23.40 ± 0.98	0
	Week 6	20.63 ± 1.35	0	20.23 ± 1.85	0
Lipase [µkat/l]	Week 0	3.54 ± 0.97	0	3.44 ± 0.73	0
	Week 6	4.20 ± 0.97	1	4.03 ± 0.80	0

Table IV.D-3 Biochemical Markers of Safety for Treatment and Placebo Groups at Baseline and Endpoint are Given and Number of Clinically Significant Values (CSV) for Subjects is Recorded

Parameters	Week	Mean ± SD	CSV	Mean ± SD	CSV
Total	Week 0		2		1
	Week 6		6		1

CSV = Clinically significant value

(ii) Studies Conducted with Other *Lactobacillus reuteri* Strains

In addition, to product specific safety information provided on *L. reuteri* in hypercholesterolemic subjects, there is a large body of clinical evidence from studies conducted in humans consuming various strains of *L. reuteri* at doses in excess of the intended use NCIMB 30242 in supplement and food products.

The GRAS self-affirmed use of *L. reuteri* DSM 17938 for use in food categories was recently Notified to the FDA (U.S. FDA, 2008). A total of 49 clinical studies were reviewed by the Notifier and FDA. Of these studies 25 studies were conducted in adults administered *L. reuteri* at doses of $1x10^8$ to $1x10^{11}$ CFU/person per day for durations of between 10 days to 6 months. The participants of these studies included healthy adults, elderly subjects with long-term constipation, subjects with irritable bowel syndrome, individuals with *Helicobacter pylori* infection, immunocompromised individuals with HIV, and ileostomized subjects. Thirteen studies investigated the effects of oral *L. reuteri* consumption in children administered $5.6x10^7$ to $1x10^{11}$ CFU/person over treatment intervals of up to 14 weeks, and 11 studies were conducted in infants consuming $1x10^8$ to $1.2x10^9$ CFU per person for periods of up to 1 year. No adverse effects or severe adverse effects attributable to *L. reuteri* were reported in any of these studies.

An updated literature search was conducted to identify human studies published since 2008. Ten new studies were identified. A tabulated summary of these studies, and those published in the literature and reviewed by FDA are presented in Appendix C. Based on the available evidence studies published within the literature to date, there is no evidence to suggest that the members of the species *L. reuteri* have pathogenic or toxicogenic potential. In all studies published in the literature, the consumption of *L. reuteri* is well tolerated, and is generally associated with beneficial effects on nutritional health.

E. Pathogenicity

There are numerous cases of *Lactobacillemia* reported in the literature; however as described in detailed reviews of *Lactobacillemia*, these cases are almost exclusively associated with serious underlying diseases or exposure *via* non-parenteral routes (Gasser, 1994; Saxelin *et al.*, 1996a). It has been suggested that probiotic infections are more likely to occur in patients with a predisposition to infection (immunocompromised or immunodeficient); however, many

Lactobacillus probiotics, including *L. reuteri*, have been safely used in subjects with diseases which could render them susceptible to infection (*e.g.*, immunocompromised HIV patients with persistent diarrhea, subjects undergoing biliary cancer surgery, patients with Crohn's disease and colitis) (Wolf *et al.*, 1998; Marteau, 2001; Salminen *et al.*, 2004; Sugawara *et al.*, 2006).

Subjects with exposed parenteral access sites may also be at increased risk for developing bacteremia in conjunction with probiotic use. Gasser reviewed the safety of lactic acid bacteria and their occurrence in human clinical infections. A total of 60 case-studies of endocarditis due to lactic acid bacteria were reviewed (Gasser, 1994). Of the 60 reported cases of endocarditis, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, and *Lactobacillus casei* were the most common organisms associated with infection. *Lactobacillus acidophilus* was identified in 2 cases of endocarditis in the review. No cases of human clinical infections with *L. reuteri* were reported by the authors. To identify clinical cases suggestive of *L. reuteri* pathogenicity, comprehensive and detailed searches of the published scientific literature were conducted by Cantox Health Sciences International through August 2010. MEDLINE®, AGRICOLA, ToxFile, Biosis Previews®, Biosis Toxline®, Food Sci. & Tech. Abstracts, CAB Abstracts, FOODLINE®, NTIS and EMBASE served as the primary sources of published literature and the following search terms were used *Lactobacillus* and *reuteri* and (infection, pathogenicity, toxicity, infectivity, meningitis, endocarditis, respiratory, bacteremia and impetigo). These database searches failed to produce literature documenting cases of *L. reuteri* infection. Based on the current long-history of use of *L. reuteri* in food (*i.e.*, probiotic products, and sourdough bread fermentation), and the fact that the species is natural commensal in many animals including humans, the absence of case-reports of infectivity strongly support that the species can be declared non-pathogenic.

Finally, Gasser also reviewed a large number of case-studies where lactic acid bacteria were associated with blood stream, chest, and digestive tract infections. The author concluded that "except for a few rare cases, there is always a severe underlying disease, and generally an obvious portal of entry" (*e.g.*, tooth extraction, cecal carcinoma). Of interest, it should be noted that in the majority of cases, the infection was associated with some form of dental surgery. Gasser also concluded that the occurrence of infections was not sufficient to change the generally recognized as safe status of lactic acid bacteria, and that rare occurrences of infection from these organisms provides insufficient evidence to suggest that their use in food poses any danger. This position is supported by the post-marketing studies reviewed by Saxelin *et al.* (1996b), where the introduction of probiotic foods to the Scandinavian market was not associated with increased incidences of lactic acid bacterial infections.

Based on the available evidence reviewed above it can be concluded that the species *L. reuteri* is a non-pathogenic species.

F. Antibiotic Resistance

Antibiotic resistance testing of probiotic organisms is necessary to ensure that antibiotic resistance determinants are not introduced into a context where these genes are at risk of being transferred to pathogenic organisms. The minimum inhibitory concentration's (MIC) of various antibiotics against *L. reuteri* NCIMB 30242 were determined using the agar dilution method in accordance with ISO 10932 guidelines (ISO 10932, 2010). The antimicrobial resistance pattern of *L. reuteri* NCIMB 30242 is presented in Table IV.F-1 below, and an assessment of antibiotic resistance was determined through comparison of the observed MIC's relative to the most recent European Food Safety Agency (EFSA) breakpoint values[6] (EFSA, 2008) (Table 4.5-1). *L. reuteri* NCIMB 30242 was shown to be susceptible to the antibiotics tested at a concentration below the cut-off MIC established by the EFSA.

Table IV.F-1 Antibiotic Resistance Profile of *Lactobacillus reuteri* NCIMB 30242 – Agar Dilution Method

Antibiotic	*L. reuteri* NCIMB 30242 µg/mL	EFSA Breakpoint values *L. reuteri* µg/mL	*Sensitive (S) Resistant (R)*
Gentamicin	1	8	S
Streptomycin	16	64	S
Tetracycline	16	16	S
Erythromycin	0.5	1	S
Clindamycin	0.25	1	S
Chloramphenicol	4	4	S
Ampicillin	1	2	S
Neomycin	2.00	8	S

Adapted from Branton *et al.* (2010).

As described in Section II.D, the genome of *L. reuteri* NCIMB 30242 has been sequenced and annotated. Although a number of known resistance features intrinsic to the species, including a multidrug efflux pump, were identified in the genome, the presence of unique antibiotic resistance determinants specific to the strain were not identified. During the annotation process no mobile genetic elements were identified. Additionally no extra chromosomal DNA was identified following repeated agarose gel analysis of DNA extracted from cell lysates, indicating the absence of plasmid DNA.

Work performed by others on the strain have reported a MIC value for chloramphenicol that was one dilution above the EFSA defined breakpoint (unpublished communication). It is not uncommon for small differences in growth conditions or variability in inoculus quantity to result in deviations in MIC values produced during testing (Egervärn *et al.*, 2007a,b). However, in light of the differing results for chloramphenicol, the genome was carefully analyzed for determinants

[6] Microbiological breakpoints are set by analyzing the Minimum Inhibitory Concentration (MIC) or inhibition zone diameters among several strains within a species. Strains that have acquired bacterial resistance genes will generally clearly deviate from the normal susceptible population and are categorized as resistant.

of chloramphenicol resistance (*i.e.*, *cat*-TC). No homologs of chloramphenicol resistance genes were identified in the annotated sequence and BLAST searches performed using published chloramphenicol acetyltranferase against the genomic sequence did not identify homologous genes encoded by *L. reuteri* NCIMB 30242. Similar analysis using sequences for transporters associated with chloramphenicol resistance also were performed. tBLASTn searches with the chloramphenicol resistance associated transporter clm did not identify homologous sequences, the chloramphenicol transporter FloR was 23% identical to the identified multidrug transporter and an e value of 4E-6. This fact, coupled with the low level of resistance and the difference in results obtained under different growth conditions suggest that the resistance observed may be the result of conditional differences in chloramphenicol uptake and not based on a specific transmissible resistance gene.

G. Additional Metabolic Properties

(i) D-Lactic Acid Production

Many probiotic strains are capable of fermenting lactose to racemic mixtures of D(-)- and L(+)-lactic acid, and as reported by Branton *et al.* (2010) *L. reuteri* NCIMB 30242 is known to ferment lactose to the above lactic acid mixture in a ratio of 45:55% (Table IV.G-1). In most individuals the presence of D-lactic acid in fermented milk products such as yoghurt does not represent a hazard risk; however, the overgrowth of commensal microorganisms capable of producing D-lactate in subjects with short-bowel syndrome and intestinal failure has resulted in incidences of D-lactic acidosis and encephalopathy (Karton *et al.*, 1987; Scully *et al.*, 1989; Hudson *et al.*, 1990). These observations in subjects with short-bowel syndrome have raised concerns about D-lactic acid producing strains and the misconception that D-lactic acid is poorly metabolized in humans. D-lactic acid is in-fact readily metabolized in humans (Ewaschuk *et al.*, 2005), and toxicity only occurs when levels of the acid saturate metabolic and elimination pathways; such conditions would not be expected in normal individuals with functional small intestines. Evidence for the safety of D-lactic acid producing strains of lactic acid bacteria in humans is supported by a large body of clinical evidence obtained with *L. reuteri* ATCC 55730. *L. reuteri* ATCC 55730 produces a racemic mixture of D- and L-lactic acid and over 35 clinical studies conducted in adults, children, and infants (15, 9, and 11 studies respectively) have not reported adverse events related to D-lactic acidosis. Specifically, Connolly *et al.* (2005) measured blood levels of D-lactic acid in 14 infants consuming $1x10^8$ CFU *L. reuteri* ATCC 55730/day from birth through 12 months of age. Following 6 and 12 months of probiotic consumption, D-lactic acid levels were within the normal range in the children.

Table IV.G-1 Quantity of D- and L-lactic Produced by Three Species of *Lactobacillus* in Modified MRS Broth after 48 Hours Incubation at 37°C[a]

Strain	D-lactate quantity (mM)	L-lactate quantity (mM)	D/L ratio
L. reuteri NCIMB 30242	47.4 ± 4.8	59.1 ± 5	9:11
L. delbrueckii bulgaricus ATCC 11842	67.7 ± 1	18.5 ± 0.2	26:7
L. rhamnosus strain GG ATCC 53103	15.7 ± 0.8	103.2 ± 10.2	13:87

[a] The ratio of D/L lactate was determined by measuring the concentration of each enantiomer in spent culture media after 48 h at 37°C with an enzymatic kit according to the manufacturer's directions (Enzyplus D/L lactic acid, Biocontrol systems Washington DC). *L. delbrueckii* bulgaricus ATCC 1182 and *L. rhamnosus* GG ATCC 53103 were used as positive controls for production of D-lactate and L-lactate respectively. Adapted from Branton *et al.* (2010)

(ii) Deconjugation of Bile Acids

Many probiotic bacteria including *Lactobacilli* and *Bifidobacteria* can actively de-conjugate bile acids, the activity of which can be important for their survival in the gut. *L. reuteri* NCIMB 30242 was specifically selected for its high bile salt activity and corresponding ability to reduce cholesterol in human and animals when consumed on a repeated basis. As described, this phenotype is believed to improve survival of the organism in the gastrointestinal tract and is therefore a common feature among probiotic bacteria, and is particularly prevalent among bacteria that are natural gut commensals. The BSH producing phenotype of *L. reuteri* NCIMB 30242 is associated with capacity of the organism to reduce cholesterol levels in animals and humans consuming the organism on a daily basis, and is therefore a desired feature of the organism. A number of organisms displaying active BSH activity are widely distributed in the marketplace in foods world-wide. For example, as reported by Marteau *et al.* (2002), *B. animalis* DN173-010 is an active producer of BSH enzymes; *B. animalis* DN173-010 is the active probiotic in Danone's Activia yogurts, and is a strain that has a long history of safe consumption world-wide (Guyonnet *et al.*, 2007).

Some bile acid deconjugation activity (7-α-dehydroxylase activity) may be harmful however if it results in the production of toxic secondary bile acids which may be linked to colon cancer (Cheah and Bernstein, 1990). Evidence for the expression of 7-α-dehydroxylase in lactic acid bacteria has not been reported in the literature, and is a genotype that appears to be limited to Enterobacter and Clostridium species within the gut microflora (Begley *et al.*, 2006). A discussion of the nutritional implications of repeated consumption of BSH active microorganisms is presented in Section IV.I.

(iii) Biogenic Amine Production

Many lactic acid bacteria exhibit amino acid decarboxylase activity. The decarboxylation of specific free amino acids results in the formation of biogenic amines, which include histamine, tyramine, putrescine, cadaverine, phenylethylamine, and agmatine; compounds that can

become toxic if consumed in quantities that exceed a metabolic threshold. Reports of toxicity from the consumption of biogenic amines are rare, and when they occur are usually associated with histamine, and to a lesser extent tyramine exposure. The adverse effects of biogenic amine exposure in healthy individuals generally involves the development of headache, palpitations, flushing, and to a lesser extent nausea, diarrhea, and erythema (Becker *et al.*, 2001; Ohnuma *et al.*, 2001; Miki *et al.*, 2005). For histamine, the hazardous intake level has been reported to be ≥50 mg/100 g of food (Lehane and Olley, 2000), and toxic physiological responses to tyramine typically involve exposures of between 10 to 80 mg/100 g of food (Edwards and Sandine, 1981). In subjects with genetic or drug-induced impairment of biogenic amine metabolism (*i.e.*, deficiencies in, or the inhibition of, enzymes catalyzing de-amination) toxicity thresholds are likely lower. For example, the toxic level for tyramine is reported to be as low as 6 mg in sensitive individuals (Edwards and Sandine, 1981).

It should be emphasized however, that physiological responses to biogenic amines is dose dependent, and exposure to these compounds is expected on a daily basis as the gastrointestinal tract contains numerous microorganisms with active amine degradation enzymatic capacity, and the presence of biogenic amines in wine, cider, cheeses, and cured meats due to the presence of lactic acid fermenting bacteria is common (Suzzi and Gardini, 2003; Ferreira and Pinho, 2006; Garai *et al.*, 2006; Landete *et al.*, 2007); consumption of these foods generally does not result in adverse effects or toxicity.

To determine the biogenic amine production capacity of *L. reuteri* NCIMB 30242, the organism was incubated overnight in MRS broth supplemented with either 0.1% (w/v) histidine or tyrosine and 0.005% (w/v) pyridoxal-5-phosphate (Branton *et al.*, 2010). The cultures were incubated for a total of 18 hours at 37°C. Each isolate was then subcultured under the same conditions described above for 3 or 5 passages to induce production of amino acid decarboxylase enzymes. A positive control culture with known histamine production also was cultivated under identical conditions. Following the final incubation, the bacteria were plated on decarboxylase indicator media developed by Bover-Cid and Holzapfel (1999) and the production of biogenic amines evaluated *via* color development. All plates were incubated for a minimum of 48 hours to allow maximal growth and color development. Using similar cultivation conditions, the culture media also was analyzed for the presence of putrescine, histamine, and cadaverine using high-performance liquid chromatography (HPLC) analysis. Under optimal conditions for the production of biogenic amines, neither the colorimetric assay, nor the HPLC analyses (Table IV.G-2) produced evidence of biogenic amine production, and *L. reuteri* NCIMB 30242 was concluded to be a non-biogenic amine producing organism.

Table IV.G-2 Quantification of Biogenic Amines in Independent Lyophilized Culture Samples of *Lactobacillus reuteri* NCIMB 30242 Detected by HPLC[a]

Amine	Sample 1	Sample 2	Sample 3
Putrescine	<1 mg/L	<1 mg/L	<1 mg/L
Cadaverine	<1 mg/L	<1 mg/L	<1 mg/L
Histamine	<1 mg/L	<1 mg/L	<1 mg/L
Tyramine	<1 mg/L	<1 mg/L	<1 mg/L

HPLC = high-performance liquid chromatography
[a] Spermine (4 ppm) was used as an internal standard, and quantities were determined based on standard curves for each compound ranging from 1.0-100 ppm generated under the same elution conditions. Adapted from Branton *et al.* (2010)

(iv) Production of Antimicrobials

The production of antimicrobial compounds by *Lactobacillus* species used in the food industry is common; nisin is a notable example, and the broad-spectrum antimicrobial function of this compound has been adopted by the food industry for use as a preservative in the U.S. (21 CFR §184.1540) (U.S. FDA, 2011a). Although the capacity to produce antimicrobial compounds by lactic acid bacteria is ubiquitous among microorganisms used in the food industry, this phenotype may represent an undesirable trait depending on the mechanism of action of the antimicrobial agent that is produced. Many clinically important antibiotics are obtained from microorganism sources, and the introduction of antibiotic producing probiotics is undesirable since widespread use in food may lead to antibiotic resistance in pathogenic microorganisms. Strains of *L. reuteri* have been reported to produce antimicrobial compounds, 2 notable examples are the bacteriocin reutericin and the antimicrobial reuterin (Kawai *et al.*, 2004; Cadieux *et al.*, 2008). The capacity of *L. reuteri* NCIMB 30242 to synthesize bacteriocins and reuterin was investigated by Branton *et al.* (2010) and is described below.

Bacteriocin Production

Many Lactobacilli are known to produce bacteriocins: small, heat-stable peptides with antimicrobial activity usually against related organisms, or organisms that may occupy the same niche. The capacity of *L. reuteri* NCIMB 30242 to produce bacteriocin-like activity was assessed by the agar well diffusion method using filter sterilized, pH-adjusted, heat treated supernatants from overnight cultures of the strain. For evidence of bacteriocin activity the following indicator organisms were co-cultured with *L. reuteri*: *Pseudomonas aeriginosa* ATCC 10145 and *Staphylococcus aureus* ATCC 43300 and *Lactobacillus delbrueckii* bulgaricus ATTC 11842. The indicator organisms were chosen from a panel commonly used for this type of testing with the exception of *L. delbrueckii. L. delbrueckii* bulgaricus was chosen because it was shown to be susceptible to the bacteriocin reutericin-6, which is produced by a human isolate of *L. reuteri* (Toba *et al.*, 1991). *Lactobacillus reuteri* NCIMB 30242 did not display inhibitory activity towards the indicator strains, suggesting that this strain of *L. reuteri* lacks bacteriocin-like antimicrobial activity.

Reuterin Production

In the presence of glycerol, many *L. reuteri* strains can synthesize 3-hydroxypropionaldehyde (3-HPA). 3-HPA is then secreted where it forms a dimer or monomeric hydrate. The various monomeric and dimeric forms of 3-HPA exist in a complex equilibrium, and this heterogeneous mixture is referred to as reuterin (Sriramulu *et al.*, 2008). Reuterin is a potent antimicrobial agent that has broad spectrum activity against Gram Positive, and Gram Negative bacteria, as well as yeasts, moulds and protozoa. The mode of action of reuterin has not been elucidated; however, it has been hypothesized that reuterin may inhibit bacterial DNA synthesis through antagonism of bacterial ribonucleotide reductase, or operate *via* inhibition of various sulfhydryl containing enzymes (Cleusix *et al.*, 2007).

To determine if further investigation into the antimicrobial activity of *L. reuteri* was required, the capacity of the strain to produce reuterin-like antimicrobial activity was assessed by comparing the inhibitory activity of *L. reuteri* NCIMB 30242 with a known reuterin producing *L. reuteri* strains (*L. reuteri* ATCC 23272). These 2 strains were grown MRS broth supplemented with 20 mM glycerol to induce reuterin production. The bacteria were then harvested, re-suspended in sterile glycerol and incubated at 37°C for 2 hours. Following this incubation, bacteria were centrifuged and the supernatant harvested, filtered and quantified for reuterin production using the methods described by Cadieux *et al.* (2008). As shown in Table IV.G-3 below, in contrast to *L. reuteri* ATCC 23272, *L. reuteri* NCIMB 30242 does not produce measurable quantities of reuterin under these test methods.

Table IV.G-3 Comparison of Reuterin Production by *Lactobacillus reuteri* NCIMB 30242 and *L. reuteri* ATCC 23272

Condition	Reuterin concentration (mM) (S.D.)
200 mM Glycerol	0
L. reuteri NCIMB 30242 incubated in 200 mM glycerol	0
L. reuteri ATCC 23272 incubated in 200 mM glycerol	107 (8)

The quantity of reuterin produced by each strain after 2-hr incubations in an aqueous 200 mM glycerol solution at 37°C as determined by OD560 using acrolein as a standard. Results shown are the means of duplicate measures from 3 samples of each condition shown ± 2 standard deviations. Adapted from Branton *et al.* (2010).

H. Bioinformatic Assessment

An overview of the bioinformatic assessment was reported in the literature by Branton *et al.* (2010). Almost 230,000 reads were generated in over 79 Mb of sequencing data. The median read length for the region was 399 bp. From the assembly, 91% of the reads have fully assembled to yield 112 large contigs (>500 bp). The size of the genome was estimated at 1.78 Mb and the depth of sequencing coverage is expected to be at least 40-fold. The contigs generated at the Innovation Centre were submitted to the RAST server (version 2.0) (Aziz *et al.*, 2008) for annotation in 6 possible open reading frames. This analysis identified 1784 coding

sequences. The seed analysis groups genes into subsystems based on functional roles such as a metabolic pathway or by class of protein (Overbeek *et al.,* 2005). Forty-four percent (44%) of the identified genes fell into established subsystems (Figure IV.H-1).

This analysis identified 16 genes that could be considered virulence genes. Four of these genes were identified as belonging to the iron scavenging mechanisms subsystem and 12 genes that were classified in the resistance to antibiotics and toxic compounds subsystem. These 12 genes included 2 genes involved in copper homeostasis, 2 genes for cobalt, zinc and cadmium resistance and 1 gene categorized as a mercuric reductase and 7 genes with motifs associated with antibiotic resistance, including a *beta*-lactamase, a MATE family multi antimicrobial extrusion protein, and 2 topoisomerase and 2 DNA gyrase genes associated with resistance to fluoroquinolones. These antimicrobial resistance elements were determined to be common to lactobacilli, and not associated with mobile elements. Therefore, these factors may be deemed part of the intrinsic resistance elements common to lactobacilli and do not represent a safety concern. No genes involved in adhesion, invasion, toxin or superantigen production or regulation of virulence were identified in this analysis.

Not in Subsystem
total (973)
non-hypothetical (552)
hypothetical (421)

In Subsystem
total (747)
non-hypothetical (712)
hypothetical (35)

- Phages, Prophages, Transposable elements (0)
- Cofactors, Vitamins, Prosthetic Groups, Pigments (88)
- Cell Wall and Capsule (57)
- Potassium metabolism (4)
- Photosynthesis (0)
- Plasmids (0)
- Miscellaneous (1)
- Membrane Transport (17)
- RNA Metabolism (30)
- Nucleosides and Nucleotides (80)
- Protein Metabolism (102)
- Cell Division and Cell Cycle (50)
- Motility and Chemotaxis (0)
- Regulation and Cell signaling (13)
- Secondary Metabolism (0)
- DNA Metabolism (41)
- Virulence (16)
- Fatty Acids, Lipids, and Isoprenoids (49)
- Nitrogen Metabolism (5)
- Dormancy and Sporulation (0)
- Respiration (19)
- Stress Response (52)
- Metabolism of Aromatic Compounds (2)
- Amino Acids and Derivatives (96)
- Sulfur Metabolism (8)
- Phosphorus Metabolism (8)
- Carbohydrates (130)

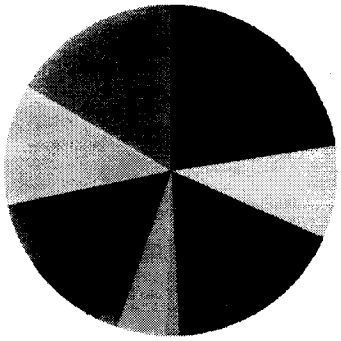

Figure IV.H-1 Annotation of *Lactobacillus reuteri* NCIMB 30242 Genome.
Subsystem distribution of identified genes in the *Lactobacillus* NCIMB 30242 genome. The draft genome sequence of *L. reuteri* NCIMB 30242 was annotated using the RAST 2.0 software and viewed using the seed viewer. 1720 genes and 64 RNA coding sequences were identified, 44% of which belonged to 1 of 196 subsystems within the 25 categories depicted above. Adapted from Branton *et al.* (2010).

I. Nutritional Impact of *L. reuteri* NCIMB 30242

(i) Effect of *L. reuteri* NCIMB 30242 on Gut Microflora

During the clinical study described in Section 5.3.1, information on the effect of twice daily consumption of *L. reuteri* NCIMB 30242 on the gut microflora composition was obtained. Fecal samples were collected at baseline and endpoint and analyzed using qPCR for total DNA, *Eubacteria*, *Bacteroides/Prevotella*, *Lactobacillus/Streptococcus/Enterococcus*, *Bifidobacterium*, *Faecalibacteria*, *Desulfovibrio*, *Enterobacteria*, *Clostridium leptum*, *Clostridium coccoides*, *Saccharomyces*, *Candida*, and *Aspergillus* (Table IV.I-1). The results show a significant decrease in *Aspergillus*, from baseline, when comparing treatment and placebo groups. All other bacterial and fungal measurements showed no significant changes between treatment and placebo groups from baseline. The change in *Aspergillus niger* observed in the study was statistically significant, however, the absolute CFU counts between groups are within the same logarithmic order of magnitude, and are therefore not expected to have any biological significance. Moreover, *Aspergillus sp.* are highly aerobic and are not natural residents of the mammalian gastrointestinal tract, which further suggests the observed change in *Aspergillus*

counts is a spurious finding. An interesting observation was trend towards lower *Clostridium* counts in the subjects consuming the *L. reuteri* supplemented yogurt, which were reduced by 4 orders of magnitude relative to the controls (P=NS). Although the biological significance of changes in colonic microflora populations is not fully understood, the current nutritional view is that reductions in *Clostridium* and *Enterobacteriaceae* due preferential growth of lactic acid bacteria is a beneficial change.

Table IV.I-1 Effect of *L. reuteri* NCIMB 30242 on Fecal Microflora[a]

	Placebo		*L. reuteri* NCIMB 30242	
	Baseline	**Week 6**	**Baseline**	**Week 6**
DNA (mg/g)	$9.7x10^{-2}$ ($1.7x10^{-3}$)	$9.5x10^{-2}$ ($2.3x10^{-3}$)	$9.6x10^{-2}$ ($2.7x10^{-3}$)	$9.9x10^{-2}$ ($1.3x10^{-3}$)
Eubacteria	$7.6x10^{10}$ ($1.6x10^{10}$)	$7.0x10^{10}$ ($1.5x10^{10}$)	$5.9x10^{10}$ ($1.2x10^{10}$)	$8.2x10^{10}$ ($2.1x10^{10}$)
Bacteroides/Prevotella	$2.2x10^{9}$ ($4.2x10^{8}$)	$8.6x10^{8}$ ($3.0x10^{8}$)	$3.6x10^{9}$ ($1.6x10^{9}$)	$8.1x10^{8}$ ($1.7x10^{8}$)
Lacto/Strepto/Entero	$7.5x10^{7}$ ($4.1x10^{7}$)	$2.7x10^{7}$ ($1.8x10^{7}$)	$9.5x10^{7}$ ($7.1x10^{7}$)	$1.2x10^{7}$ ($3.5x10^{6}$)
Bifidobacteria	$3.4x10^{8}$ ($1.9x10^{8}$)	$2.5x10^{7}$ ($8.7x10^{6}$)	$2.8x10^{8}$ ($8.2x10^{7}$)	$3.7x10^{7}$ ($1.1x10^{7}$)
Faecalibacteria	$5.1x10^{9}$ ($1.6x10^{9}$)	$3.0x10^{9}$ ($1.2x10^{9}$)	$3.1x10^{9}$ ($5.7x10^{8}$)	$4.4x10^{9}$ ($1.1x10^{9}$)
Enterobacteriaceae	$2.2x10^{7}$ ($1.1x10^{7}$)	$1.5x10^{7}$ ($1.1x10^{7}$)	$1.1x10^{7}$ ($6.4x10^{6}$)	$6.2x10^{6}$ ($4.8x10^{6}$)
Saccharomyces	$9.3x10^{4}$ ($6.4x10^{4}$)	$6.7x10^{4}$ ($1.8x10^{4}$)	$2.3x10^{4}$ ($6.1x10^{3}$)	$7.7x10^{4}$ ($3.5x10^{4}$)
Clostridium coccoides	$4.6x10^{9}$ ($6.8x10^{8}$)	$2.3x10^{9}$ ($4.6x10^{8}$)	$6.7x10^{9}$ ($2.3x10^{9}$)	$2.9x10^{4}$ ($6.0x10^{8}$)
Candida	$7.9x10^{4}$ ($5.0x10^{4}$)	$8.0x10^{3}$ ($4.8x10^{3}$)	$2.9x10^{4}$ ($1.1x10^{4}$)	$1.1x10^{4}$ ($6.5x10^{3}$)
Clostridium leptum	$2.0x10^{10}$ ($2.7x10^{9}$)	$8.1x10^{9}$ ($1.7x10^{9}$)	$2.1x10^{10}$ ($4.6x10^{9}$)	$8.8x10^{9}$ ($1.3x10^{9}$)
Desulfovibrio	$4.5x10^{8}$ ($7.2x10^{7}$)	$4.2x10^{8}$ ($9.9x10^{7}$)	$5.0x10^{8}$ ($1.3x10^{8}$)	$4.9x10^{8}$ ($1.2x10^{8}$)
Aspergillus[b]	$2.7x10^{5}$ ($1.8x10^{5}$)	$8.5x10^{4}$ ($6.4x10^{4}$)	$2.6x10^{8}$ ($2.0x10^{5}$)	$4.8x10^{4}$ ($1.2x10^{4}$)

Data presented as mean followed by standard error in parentheses
[a] Fecal assessment using total DNA, bacterial species concentration in CFU/g
[b] Statistically significant between group difference at week 6 (P<0.05)

(ii) Effect of *L. reuteri* NCIMB 30242 on Bile Salt Metabolism

As discussed in Section 5.6.2, *L. reuteri* NCIMB 30242 is a BSH-active microorganism. Microbial mediated deconjugation of bile acids is a common phenotype among gut microorganisms, and in humans, this bacterial biotransformation event is initiated within the mid-ileum and is completed in the colon (Hofmann, 2009a). Based on the presumed viability and transient residence of *L. reuteri* NCIMB 30242 within the small intestine, and the corresponding effects on cholesterol metabolism that have been observed during regular dietary consumption of the strain by rodents and humans – an effect that is believed to be related to the bile-salt hydrolase phenotype of the organism – the nutritional impact of regular consumption of a bile salt active microorganism is considered. Since bile salt deconjugation activity is largely restricted to bile-salt active microbes within the colon, a specific emphasis has been placed on evidence characterizing the effect of bile acid deconjugation proximal to the terminal ileum, and the physiological consequences of this action on human nutrition and safety.

Background

Bile consists of bile acids, cholesterol, phospholipids, and the hemoglobin breakdown product biliverdin. Bile is synthesized in the liver, and following production is secreted into the biliary ductules for transport to the gall bladder for storage. The consumption of a meal results in the secretion of cholecystokinin, which in turn stimulates the gall bladder to contract releasing bile into the duodenum. Bile salts are the principal component of bile and are synthesized from cholesterol in the pericentral hepatocytes *via* a series of complex chemical reactions. The primary bile acids produced by the liver in humans are cholic acid, chenodeoxycholic acid, and ursodeoxycholic acid (Figure IV.I-1). Following their synthesis and prior to secretion in biliary ductules, the bile acids are conjugated to the amino acids glycine or taurine. The corresponding conjugated molecules are denoted using the pre-fix tauro- and glyco. Bile acid conjugation results in the conversion of the bile acid from a weak acid to a strong acid, and under the physiological pH of the small intestine these molecules are fully dissociated amphiphatic molecules (Hofmann, 2009b). Bile acid conjugation serves a number of important physiological functions. First, the amphiphatic nature of these molecules imparts detergent properties to the bile, which assists with fat digestion by emulsification of fat globules, and promotion of mixed micelle formation. Bile acids also facilitate the incorporation of fat soluble vitamins, cholesterol and other lipophilic compounds into mixed micelles, and therefore also influence the absorptive efficiency of the small intestine to these compounds. Conjugation increases the water solubility of the bile acids at neutral pH, and prevents passive absorption of the bile acid in the small intestine. Finally, bile acid conjugation facilitates their efficient enterohepatic circulation, which occurs *via* active transporters (ASBT/OSTα/OSTβ system) in the terminal ileum that display high affinity for the conjugated form of the molecules (Hofmann, 2009a).

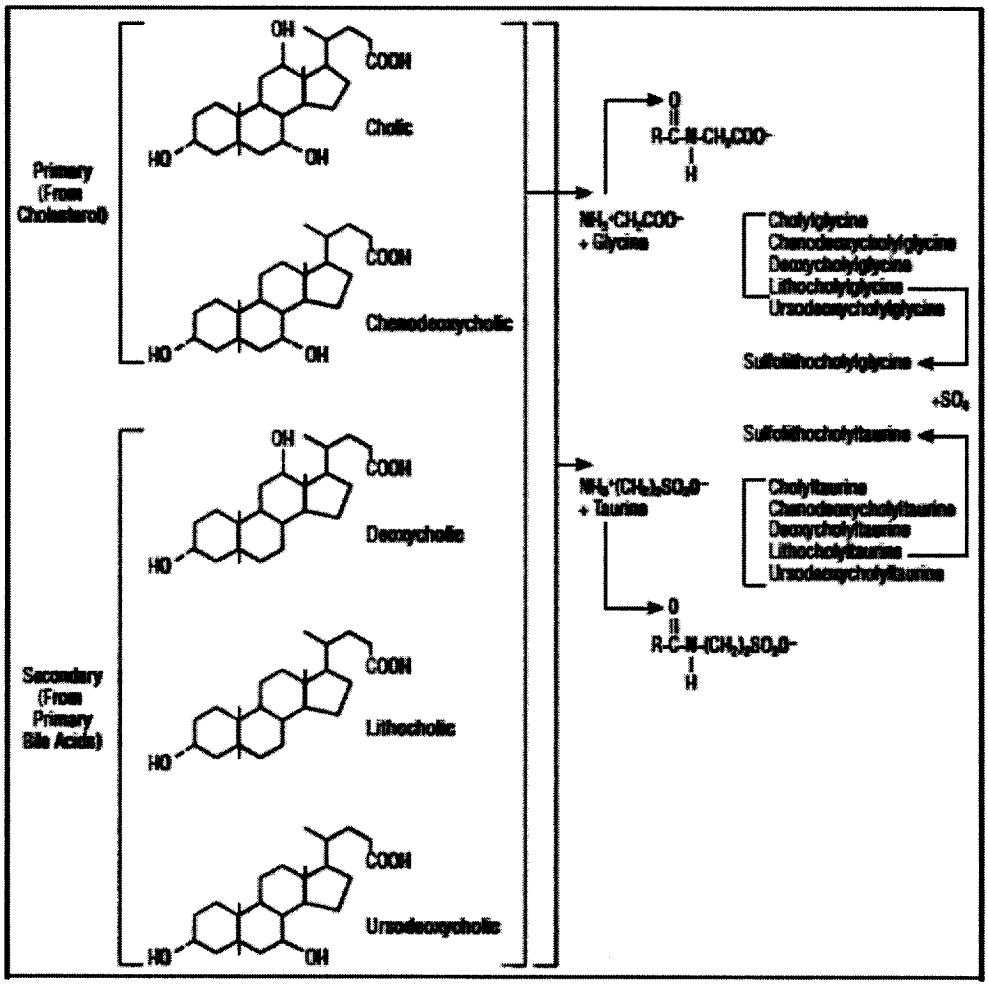

Figure IV.I-1 Bile Acid Metabolism – Illustration of major primary and secondary bile acids in humans

(Adapted from Hofmann, 2009a).

Under normal conditions, the process of enterohepatic circulation ensures that the majority of bile acids secreted in the duodenum are effectively scavenged, and 97% of the conjugated bile acids secreted by the gall bladder are re-absorbed (Hofmann, 2009b). The remaining 3% of conjugated bile acids that escape re-absorption are subject to metabolism by the indigenous microflora of the intestine. In humans, bacterial metabolism of bile acids begins in the mid-ileum and is completed in the colon (Hofmann, 2009a). In rodents, deconjugation is initiated in the proximal small intestine (Hofmann, 2009a). Bile acids reaching the gut microflora are then subject to several biotransformations, which are dominated by deconjugation, 7α/β dehydroxylation, and C-7 epimerization reactions (Ridlon *et al.*, 2006). Other biotransformations include oxidation at C-3 or C-7, and epimerization at C-7; however, these transformation events are limited and have no known physiological significance (Ridlon *et al.*, 2006; Hofmann, 2009a).

Deconjugation of bile acids is mediated by a number of microbial BSHs, and expression of this enzyme is a common phenotype among gut microflora: various *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Clostridium*, and *Bacteroides* species are known to display varying levels of bile acid deconjugation activities (Ridlon *et al.*, 2006). There are numerous isoenzyme forms of the enzyme expressed among the various species within the colon, and each enzyme exhibits varying activity, optimal functional pH range, and affinity towards different conjugated bile acids (Ridlon *et al.*, 2006). Although the functional role(s) of BSH expression by microorganisms within the gastrointestinal tract are unclear, the general consensus is that BSH activity is positively correlated with the capacity of a microbe to colonize and survive within the environment of the gastrointestinal tract (Ridlon *et al.*, 2006). For example, using metagenomic analysis, Jones *et al.* (2008) demonstrated that functional BSH expression can be found among all major bacterial divisions and archeal species of the gut, and that the BSH genotype is enriched in the human gut microbiome. Overexpression of BSH in *Listeria innocua*, an organism that does not contain BSH within its genome, provided a distinct survival advantage to the organism over the wild-type strain (Jones *et al.*, 2008). Microbial deconjugation of bile acids is complete in the large-intestine, and as discussed, the corresponding free bile acids are then subject to secondary dehydroxylation, oxidation, and epimerization transformations.

The production of secondary bile acids is significant in the colon, and secondary bile acids are the predominate bile acids present in human feces, an interesting observation given the microorganisms displaying 7 α-dehydroxylation reactions are limited to a few species (*Clostridium*, *Enterococcus*, *E. coli*, *Bacteroids*) constituting only ~0.0001% of the total colonic microflora (Ridlon *et al.*, 2006). The 7 α-dehydroxylation reactions are the most quantitatively important bacterial bile salt biotransformation in the human colon, and the 7-deoxy bile acids deoxycholic acid (DCA) and lithocholic acid (LCA) – produced from dehydroxylation of cholic and chenodeoxycholic acid respectively – represent the predominant bile acid metabolites in the colon. Both DCA and LCA are more hydrophobic than their primary counterparts, and as weak acids display reduced solubility at low pH. Because, these secondary bile acids are more lipophilic, small amounts are passively absorbed into the portal circulation through the gut mucosa (see background on bile acid kinetics below). Deoxycholic acid and LCA can be passively absorbed through the colon into the portal circulation and transported to the liver where they are re-conjugated to glycine or taurine and excreted in the bile in a similar fashion to their primary counterparts. In addition to conjugation, LCA acid undergoes a further sulfation reaction, which prevents further enterohepatic circulation from taking place and the sulfated molecule is excreted in the feces (Ridlon *et al.*, 2006). A fecal elimination pathway for secondary bile acids is important as the hydrophobic nature of these dehydroxylated molecules render them toxic to cellular membranes at high concentrations, and LCA is highly hepatotoxic in experimental animals (Hofmann, 1999; Beilke *et al.*, 2008). Deconjugated bile acids are toxic to colon mucosal cells, and LCA has been reported in a number of animals studies to promote liver and colon carcinogenesis (Stalker *et al.*, 1994 ; Baijal *et al.*, 1998; Kozoni *et al.*, 2000). Because, bile acid production is increased during the consumption of high fat diets, DCA and

LCA have therefore been implicated as the endogenous etiologic agents in the epidemiological association between fat intake and the incidence of colon cancer among consumers of high fat diets (Bernstein *et al.*, 2009). Definitive evidence substantiating the association between exposure to secondary bile acids and the development of various has yet to be produced. Dietary patterns that result in increases in bile salt production, bile acid excretion, and a corresponding increase in the production of toxic secondary bile acids may therefore be undesirable.

Consequences of Bile Acid Deconjugation in the Gastrointestinal Tract

Probiotic Microorganisms and Bile Salt Hydrolase Activity

As discussed above, although most bile salt transformations take place in the colon, microorganisms that preferentially grow in the small intestine also contribute to the total pool of deconjugated bile acids. It has been suggested that colonization of the small intestine with a microorganisms that display high BSH activity may lead to increased excretion of bile acids in the feces, an effect that is undesirable since deconjugated bile acids are subject to biotransformation by 7-α-dehydroxylase active microorganisms leading to the increased production of toxic secondary bile acids that have been implicated as the etiologic agents in cancers of the gastrointestinal tract, and gall stone disease. Thus, the consumption of BSH active microorganisms that are viable and active within the small intestine, may simulate the physiological effect of a high-fat diet, which results in increased colonic loads of bile acids, and increased local and systemic exposure to DCA and LCA. This viewpoint and the corresponding importance of characterizing bile-salt hydrolase activity during the safety evaluation of a probiotic has been widely reported in several probiotic safety reviews, and also can be found in guidance documentation from various Regulatory Authorities (Salminen *et al.*, 1998; FAO/WHO, 2002; Gueimonde *et al.*, 2004; Health Canada, 2005, 2006; Begley *et al.*, 2006; Bernardeau *et al.*, 2006, 2008; Pineiro and Stanton, 2007; Snydman, 2008; Wassenaar and Klein, 2008). Despite these multiple viewpoints, Micropharma has noted that to date, these concerns are entirely theoretical and have not been based on comprehensive reviews of the subject, and in some cases are based on incorrect assumptions about the physiology of bile acid metabolism.

That extensive pre-ileal deconjugation of bile salts will result in increased fecal loads of secondary bile acids is based on a common misconception that "deconjuged bile salts are less efficiently re-absorbed than their conjugated counterparts, which results in the excretion of larger amounts of free bile acids in feces." (Begley *et al.*, 2006). This concept is referenced extensively in the literature (De Rodas *et al.*, 1996; Brashears *et al.*, 1998; De Smet *et al.*, 1998; De Boever *et al.*, 2000; St-Onge *et al.*, 2000; Hatakka *et al.*, 2008; Kaushik *et al.*, 2009; Ooi *et*

al., 2010)[7]. These viewpoints are inconsistent with our current understanding of gastrointestinal physiology and evidence characterizing the metabolic fate of bile acids. These statements also were determined to lack suitable supporting references.

It has long been recognized from early studies of bile acid absorption in humans and rodents that deconjugated bile acids are efficiently absorbed throughout the small intestine *via* passive and active transport mechanisms (Schiff *et al.*, 1972; Krag and Phillips, 1974). For example, using *in vitro* intestinal incubation methods and *in vivo* perfusion studies in bile duct cannulated Sprague-Dawley rats (200 g), Schiff *et al.* (1972) evaluated the active and passive absorption of several conjugated and deconjugated bile acids. As shown in Table IV.I-2 below, the authors observed that cholic acid, deoxycholic acid, chenodeoxycholic acid, and lithocholic acid were efficiently absorbed across the proximal intestine *via* passive mechanisms. Analyses by the authors of additional data obtained from the jejunum, ileum and colon indicated that "the permeability characteristics of these regions with respect to passive bile acid absorption are essentially identical." In contrast, their corresponding conjugated counterparts displayed poor absorption within this region of the intestine. The formation of mixed micelles by conjugated bile acids further reduced their passive absorption. The authors also evaluated the apparent kinetic characteristics of the active transport system for bile acids across the ileum of the rat (Table IV.I-3). Active transport of both the conjugated and unconjugated bile acids was observed. Although deconjugation of the bile acids resulted in high transport efficiencies, the conjugated molecules displayed a higher (~2 fold) affinity for the transporter, and would be preferentially absorbed. "thus the *km for all of the conjugated bile acids are about half the value of the unconjugated bile acids. Hence, it is of interest that this active ileal transport system apparently absorbs most avidly those bile acids that are least absorbed by passive mechanisms so that the active and passive systems complement one another and bring about nearly complete absorption of bile acid from the small intestinal contents." (Schiff *et al.*, 1972). Based on these studies it can be concluded that deconjugation of bile acids proximal to the terminal ileum (region of active transport of bile acids) does not disrupt enterohepatic circulation of bile acids through their loss in feces in rodents.

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[7] "Thus, the deconjugation of bile acids in the small intestine could result in greater excretion of bile acids from the intestinal tract, especially because free bile acids are excreted more rapidly than the conjugated bile acids (De Rodas *et al.*, 1996).
"Deconjugated bile salts are less well absorbed in the enterohepatic circulation and thus are more likely to be excreted in the feces." (Brashears *et al.*, 1998).
"The ingestion of lactic acid bacteria containing active BSH results in proportions of deconjugated free bile salts, which are less water soluble and are more easily excreted *via* the feces" (De Smet *et al.*, 1998).
"Based on literature data, it was assumed that enhanced bile salt hydrolysis would make more deconjugated bile salts available for 7α-hydroxylation. This would lead to a higher concentration of secondary bile salts which are (geno)toxic and mutagenic compounds." (De Boever *et al.*, 2000).
"Deconjugated blie acids are not well absorbed by the gut mucosa and are excreted." (St-Onge *et al.*, 2000)
"Significant bile salt hydrolysis proximal to the terminal ileum reduces bile salt uptake, leading to enhanced excretion in faeces." (Hatakka *et al.*, 2008).
"The deconjugation of bile salts leads to decreased solubility and hence lower reabsorption in the enterohepatic system" (Kaushik *et al.*, 2009).
"The deconjugation of bile results in the production of deconjugated bile, which is less absorbed in the intestine and thus secreted in the feces." (Ooi *et al.*, 2010).

Table IV.I-2 Rates of Passive Absorption of Bile Acids Across the Jejunum of the Sprague-Dawley Rat

Bile acid	Apparent permeability coefficients (ΔJ/ΔCm) pmoles/min/cm/mM		
	Ionized molecule Conc.<CMC	Protonated molecule Conc.<CMC	Ionized bile acid in micelles
Cholic acid	-	**1543±157**	-
Glycocholic acid	51±4	-	69±16
Taurocholic acid	39±2	-	80±15
Deoxycholic acid	-	3795±718	-
Glycodeoxycholic acid	133±9	-	125±48
Taurodeoxycholic acid	114±7	-	115±16
Chenodeoxycholic acid		3900±512	-
Glycochenodeoxycholic acid	142±18	-	166±38
Taurochenodeoxycholic acid	138±9	-	139±41
Lithocholic acid		9600	-
Glycolithocholic acid	429±61	-	-
Taurolithocholic acid	387±39	-	-

CMC = critical micelle concentration
Adapted from Schiff *et al.* (1972)

Table IV.I-3 Apparent Kinetic Characteristics of the Active Transport System for Bile Acids Across the Ileum of the Sprague-Dawley Rat

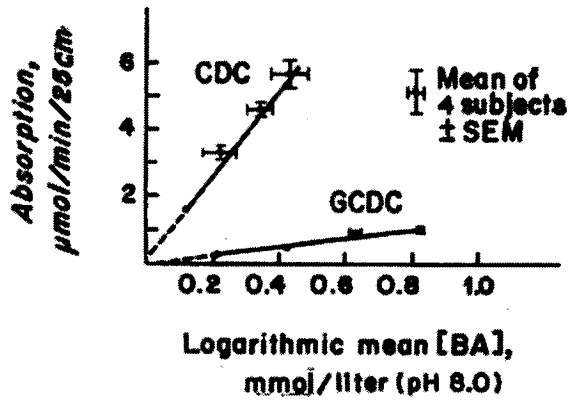
Bile acid	V_{max}	Km
Cholic acid	1906±462	0.49±26
Glycocholic acid	1543±326	0.18±0.10
Taurocholic acid	1629±236	0.23±0.07
Deoxycholic acid	224±169	0.37±0.16
Glycodeoxycholic acid	114±32	0.16±0.20
Taurodeoxycholic acid	397±27	0.19±0.04
Chenodeoxycholic acid	512±150	0.38±0.08
Glycochenodeoxycholic acid	173±40	0.21±0.01
Taurochenodeoxycholic acid	337±13	0.21±0.06
Glycolithocholic acid	45±8	0.09±0.04
Taurolithocholic acid	57±21	0.09±0.07

Adapted from Schiff *et al.* (1972)

The active and passive absorption of bile acids in humans was evaluated by Krag and Phillips (1974). The studies were conducted in 12 healthy volunteers (9 men aged 21 to 45 years, and 3 post-menopausal women). Each individual was subject to perfusions of conjugated and/or unconjugated bile acids in 25 cm intestinal segments within the distal 35 cm of the ileum or proximal jejunum. Subjects were perfused at a rate of 10 mL/minute, and samples were collected 25 cm distally by intermittent suction in the ileum and siphanage in the jejunum. As shown in Figure IV.I-2 below, the authors observed that free bile acids are absorbed faster than

their conjugated counterparts within both the jejunum and ileum. The authors further comment that the "data from man and rat are in general agreement" Krag and Phillips (1974).

A – Ileum



B – Jejunum

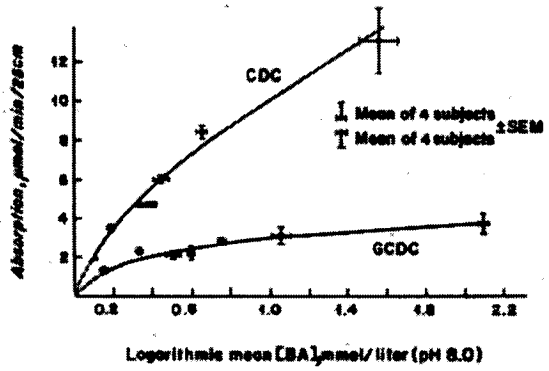


Figure IV.I-2 Kinetics of bile acid absorption in humans

A. Absorption of chenodeoxycholic acid and glycochenodeoxycholic acid in the jejunum of 4 subjects perfused with increasing concentrations of each bile acid. **B.** Absorption of chenodeoxycholic and glycochenodeoxycholic acid in 4 subjects with increasing concentrations of each bile acid (closed circles). Open circles denote subjects perfused with equimolar mixtures of both bile acids. Data represents mean ± standard error of mean. Adapted from Krag and Phillips (1974).

Additional evidence that increased microbial deconjugation of bile acids within the small intestine does not result in significant excretion of unconjugated bile acids in the feces is supported by observations from patients with intestinal stasis, and other pathologic conditions that promote the overgrowth of bacteria in the small intestine. In these individuals, the unconjugated bile acids that are formed are passively absorbed from the small intestine leading to decreased intraluminal concentrations and impaired micelle formation (Hofmann, 1999). In addition, there is a single reported case in the literature of an individual absent bile acid conjugation. In this subject, passive absorption of the unconjugated bile acids resulted in

reduced intraluminal concentrations of bile acids below that required for micelle formation, which manifested in market impairment of fat absorption. Finally, the oral administration of chenodeoxycholic acid has been used for the medical treatment of gallstones. In a study conducted by Ponz de Leon *et al.* (1980), the intestinal solubilization, absorption, pharmacokinetics and bioavailability of chenodeoxycholic acid were evaluated. Absorption of chenodeoxycholic acid was determined following duodenal infusions of ^{3}H labeled bile chenodeoxycholic acid in 5 subjects at successive doses of 250, 500, and 750 mg. 14-C polyethylene glycol or bromsulphthalein were added to the infusion doses and served as non-absorbable markers. The authors reported that absorption of duodenally-infused chenodeoxycholic acid was 96 to 99% complete.

Administration of Bile Salt Active Microorganisms to Animals

Studies Conducted Using Rodents

Park and colleagues (2007) evaluated the effect of dietary inclusion of *Lactobacillus acidophilus* ATCC 4321 on cholesterol metabolism in rats. The study was conducted using 36 male Sprague-Dawley rats (241.6±2.1 g) randomized to 1 of 4 dietary treatments containing 9 animals per group: Normal diet; hypercholesterolemic diet; Normal diet + *L. acidophilus* ($2x10^{6}$ CFU/day); hypercholesterolemic diet + *L. acidophilus* ($2x10^{6}$ CFU/day). Animals consumed their respective diets for a total duration of 21 days and measures of fecal acid sterol concentrations were obtained at the end of the treatment period. As shown in Table IV.I-4 below, statistically significant increases in concentrations of fecal secondary bile acids was observed in animals consuming the *L. acidophilus* supplemented diets. Although an apparent increase in secondary bile acid production was observed in the animals administered *L. acidophilus* in this experiment, a review of the analytical data used for analysis of fecal bile acids, and the quantitative distribution of major bile acids reported in the table below suggest inadequacies in the analytical method that render the data uninterruptable. For example, the analytical method reported by the authors was based on the method described by Grundy *et al.* (1965). This method is inappropriate for use in the analyses of rodent bile acids since the method does not allow for the detection of hyodeoxycholic acid, the predominant secondary bile acid produced within the rodent colon (Madsen *et al.*, 1976). In addition, the ratios of lithocholic acid to deoxycholic acid reported in this study appear to be in error with that expected in rats as lithocholic acid is typically identified as a minor secondary bile acid in the rodent colon, and is usually present at concentrations that are an order of magnitude below the concentrations of deoxycholic acid (Madsen *et al.*, 1976; Eyssen *et al.*, 1999). There also is no explanation for ~25 to 50% reductions in cholic acid and chenodeoxycholic acid that were reported in the study; it is difficult to envision a scenario where bile salt hydrolysis within the small intestine results in an increased excretion of bile acids into the colon manifesting in opposing directional changes in fecal concentrations of primary and secondary fecal bile acids.

Table IV.I-4 Fecal Acid Sterols (mg/day)

Diet	CA	CDCA	DCA	LCA	MCA	UBA	Total	P/B
ND	2.28[b]	1.30[b]	6.23[c]	9.88[c]	3.18[b]	1.82	25.41[d]	0.65
ND-La	1.70[c]	0.85[c]	6.88[b]	11.60[b]	11.60[b]	1.74	26.58[c]	0.71
HD	2.73[a]	1.55[a]	7.20[b]	10.35[c]	10.35[a]	1.93	29.70[b]	0.62
HD-La	1.43[d]	1.38[b]	7.90[a]	14.93[a]	14.93[a]	1.82	33.20[a]	0.71
SEM	0.13	0.05	0.13	0.52	0.52	.015	0.62	0.01

Adapted from Park *et al.* (2007)
[1] CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; MCA, α-, β-, and ω-muricholic acid; UBA, unidentified bile; P/B, Secondary bile concentration per total bile acid; ND, Normal diet; ND-La, Normal diet+*L. acidophilus* ATCC 43121; HD, Hypercholesterol diet; HD-La, Hypercholesterol diet+*L. acidophilus* ATCC 43121. SEM: Standard error of the means.
[abc] Mean values in the column with different superscripts are significantly different as determined by GLM and Duncan's multiple range tests ($P<0.05$).

The effect of administration of *Lactobacillus gasseri* on serum lipids and fecal steroids in hypercholesterolemic mice was reported by Usman and Hosono (2000). Twenty-four (24) male Wistar rats (8 weeks of age) were randomized to 1 of 4 groups: 1) cholesterol rich diet + water; 2) cholesterol rich diet + skim milk; 3) cholesterol rich diet + non-fermented milk supplemented with *L. gasseri* SBT0270; or 4) cholesterol rich diet + non-fermented milk supplemented with *L. gasseri* SBT0274. No water was administered to animals randomized to the milk groups. Animals consumed their respective diets for 14 days, and total serum and fecal bile acids were measured at the end of the treatment period using a colorimetric enzymatic test kit. At the end of the treatment period a significant reduction in serum bile acids (-68%; $p<0.05$), and a corresponding increase in total fecal bile acids (+39%; $P<0.05$) was observed in the *L. gasseri* SBT0270 group relative to the skim milk control group. No significant differences between the remaining 3 groups were noted. Both *L. gasseri* strains used in the experiment were noted by the authors as bile salt active microorganisms able to survive gastrointestinal transit; the authors were unable to explain the conflicting results observed between the 2 *L. gasseri* strains used in the experiment. Although the results of this study provide some evidence that dietary consumption of BSH active microorganisms may result in fecal loss of bile acids in rats, interpretation of the study findings is complicated by fact that fecal loss of bile salts was not observed in both BSH active strains, and the fact that the microorganisms were administered in milk, which was the sole source of fluids for the animals. Deconjugated bile acids display a high affinity to calcium salts, and the ability of high dietary concentrations of calcium salts to reduce bile salt bioavailability in animals is well established [see De Rodas *et al.* (1996) below]. Finally, the analysis method employed by the authors for quantitation of bile acids was based on the use of an enzymatic assay that uses 3 α-hydroxysteroid dehydrogenase for colorimetric quantification of bile acids, which has not been validated for measurement of bile acids under the conditions of this experiment. Due to the use of a non-qualitative analyses method, it is unclear if the authors are accurately measuring fecal bile acids or an interfering cholesterol metabolite produced by the microorganism. For example, as reported by Ridlon *et al.* (2006), 3 α-hydroxysteroid dehydrogenase activity is prevalent among intestinal bacteria.

In a subsequent study by the authors, the dose responsive effects of *L. gasseri* were evaluated under comparable study conditions reported above. *L. gasseri* SBT0270 was administered in the diet as a freeze-dried powder to rats consuming a hypercholesterolemic diet at doses of 0, $1x10^7$, $1x10^8$, or $1x10^9$ cells/rat/day for 14 days. Analyses of effects on fecal bile acids was conducted using a colorimetric test kit, and was limited to analyses on the high-dose animals only. The authors reported significant decrease (42%; $P<0.05$) in serum total bile acids, and a corresponding increase (42%; $P<0.05$) in fecal bile acids in the high-dose *L. gasseri* group relative to the controls. Similar to the previous experiment interpretation of the findings are complicated by limitations of the analytical method used for measurement of bile acids (Usman and Hosono, 2001).

Jeun *et al.* (2010) reported that increased bile acid excretion was responsible for the hypocholsterolemic effects observed in rats administered *Lactobacillus plantarum* (*L. plantarum*) *via* the oral route. Twenty-one (21) 6-week-old male C57BL/6 mice were randomized to 1 of the following 3 groups (n=7): standard commercial chow diet (control); standard chow diet and viable *L. plantarum* KCTC3928 ($1x10^9$ CFU/animal/day); or a standard chow diet and dead *L. plantarum* KCTC3928 ($1x10^{10}$ CFU/animal/day). It could not be determined if the *L. plantarum* treatments were administered *via* oral gavage or in the diet. Treatments were maintained for 4 weeks, and in addition to indices of cholesterol metabolism, measurements of fecal bile acids were obtained upon completion of the treatment period using the 3 α-hydroxysteroid dehydrogenase colorimetric assay. Hypocholesterolemic effects were reported in the animals administered the viable *L. plantarum* treatments; LDL cholesterol and plasma triglycerides reduced by 42 and 32% respectively ($P<0.05$). The authors also reported that fecal bile acids were increased by 45% in the animals administered the viable *L. plantarum* treatments. However, it was noted that the increase in fecal bile acids reported by the authors was not statistically significant. In addition, the authors do not discuss the observation that a comparable ~40% increase in fecal bile acids also was observed in the animals randomized to treatment with dead *L. plantarum*. The author's conclusion that the apparent hypocholesterolemic effect of *L. plantarum* consumption was a result of "increased bile acid excretion" is not supported by the experimental data, and the data more appropriately supports the finding that BSH activity was not correlated with increased excretion of bile acids.

Kumar *et al.* (2011) reported the hypocholesterolemic effects of *Lactobacillus plantarum* (*L. plantarum*) strains in Sprague-Dawley (SD) rats. Thirty (30) adult male SD rats (150±10 g) were randomized to 1 of 5 treatment groups administered a normal control diet, a hypercholesterolemic diet, a hypercholesterolemic diet + *L. plantarum* Lp91 ($1x10^8$ CFU/g), a hypercholesterolemic diet + encapsulated *L. plantarum* Lp91 ($1x10^8$ CFU/g), or a hypercholesterolemic diet + *L. plantarum* Lp21 ($1x10^8$ CFU/g) for a period of 3 weeks. Fecal cholic acid concentrations were measured from fecal samples obtained at baseline and at the end of the treatment period using chemical (colorimetric based) analyses. The authors reported that the combination of a hypercholesterolemic diet and *L. plantarum* consumption (all strains

tested) resulted in a several-fold increase in fecal concentrations of cholic acid above levels observed in the animals randomized to both the normal and hypercholesterolemic control diets. The results of this experiment are difficult to interpret since the fecal bile acid analysis was limited to measurement cholic acid alone, thus important information on the relative concentrations of primary to secondary bile acids is not available. Also since the colorimetric method used by the authors is not qualitative or validated, it is unclear if the authors were measuring cholic acid or an unknown *L. plantarum* cholesterol metabolite(s).

Studies Conducted Using Pigs

A number of studies in which the consumption of BSH active microorganisms on bile acid absorption were identified in the literature, and are presented below. These studies were conducted using a number of pig feeding models, and the similarity of the pig gastrointestinal anatomy and physiology to that of humans makes this model suitable for extrapolation of findings to humans (Cooper *et al.*, 1997).

Bifidobacterium animalis DN-173 010 is the probiotic microorganism added to Danone's popular Activia brand of yogurts. Several studies have shown that the organism exhibits a high survival capacity throughout the small intestine and colon (Pochart *et al.*, 1992; Chen *et al.*, 1999; Fujiwara *et al.*, 2001), and *in vitro* studies have shown that DN-173 010 displays significant BSH activity (Lepercq *et al.*, 2004). In addition, regular consumption of fermented milks containing the organism also has been shown to reduce colonic transit time in healthy women (Marteau *et al.*, 2002). The combination of reduced colonic transit and persistence of this BSH active microorganism in the small intestine are conditions that would be expected to result in significant increases in pre-ileal bile salt deconjugation. Lepercq *et al.* (2004) have evaluated the effect of repeat administration of DN-173 010 on the enterohepatic circulation of bile salts in castrated Large White pigs. Groups of 6 healthy animals were administered live or inactivated doses ($3.5x10^{11}$ CFU twice daily) of DN-173 010 for 15 days. At time = 0, following 1 and 2 weeks of treatment portal serum samples were collected over a 6-hour postprandial period to evaluate the concentrations of total and unconjugated bile acids. Bile acids were quantified using gas liquid chromatographic analyses. After 2 weeks of treatment, the concentration of serum unconjugated bile acids was significantly increased by 50% relative to controls. However, no change in total bile acid concentrations (conjugated + unconjugated) was noted (84.6 *vs.* 87.0 µmol/L; controls *vs.* treatment). Consistent with experimental data on the absorption of bile acids in rodents and humans discussed above, the presence of microbial deconjugation activity proximal to the terminal ileum did not alter enterohepatic circulation of bile acids. In this experiment deconjugated bile acids, produced *via* BSH active microorganisms residing in the small intestine, were readily absorbed.

In subsequent study by the authors, the feasibility of increasing ursodeoxycholic acid (UDCA) in the enterohepatic circulation of pigs by the oral administration of 2 enzymatically active microorganisms was assessed (Lepercq *et al.*, 2005). The authors administered the bile salt

hydrolyzing bacteria *Bifidobacteria animalis* DN 173-010, and *Clostridium absonum* (*C. absonum*) ATCC 27555, which is capable of epimerizing chenodeoxycholic acid (CDCA) to UDCA. To evaluate the effect of dietary intervention on enterohepatic circulation of bile acids, 12 castrated Large White pigs (41.3±2.0 g) were subject to surgical cannulation of the portal and jugular veins, including implantation of lower choledocal and upper duodenal catheters. Following recovery from surgery, animals were paired, and 1 of each pair was randomly assigned to 1 of 2 treatment groups receiving a combination of living or heat inactivated *B. animalis* DN 130-010 and *C. absonum* ATCC 27555. *B. animalis* DN 173-010 ($3.5x10^{11}$ CFU/animal) and *C. absonum* ATCC 27555 ($2.2x10^{11}$ CFU/animal) were administered as 2 daily intraduodenal infusions prior to each meal for a duration of 3 weeks (Days 14 through 35), and animals were maintained on hypercholesterolemic diets throughout the entire experimental period (Days 0 to 42). Measurements of bile acid absorption into the portal vein were obtained on Days 14, 21, 28, 35, and 42, using gas liquid chromatographic analyses. Administration of viable microorganisms resulted in statistically significant increases in portal vein concentrations of deconjugated bile acids, which were increased by 62 to 75% throughout the treatment period. Consistent with the epimerization activity of *C. absonum* ATCC 27555, portal concentrations of UDCA were increased several fold in the animals consuming the viable microorganism only. No between group difference in portal concentrations of total bile acids was observed. A notable finding was a significant between group differences in fecal concentrations of lithocholic acid (LCA) over time. As expected, consumption of the high-fat diets resulted in statistically significant increases in LCA in the animals consuming the heat inactivated bacteria, which increased from 27.3% of total fecal bile acids on Day 14 to 43.6% of total fecal bile acids on Day 42 ($P<0.05$). In contrast, no changes in fecal bile acid concentrations of LCA were observed with time in the pigs administered the viable microbes. The absence of time dependent increases in LCA is consistent with the "pre-mature" absorption of deconjugated bile acids in the small intestine, preventing their exposure to dehydroxylating microbes within the colon. This experiment provides strong evidence that the residence of bile salt active microorganisms within the small intestine does not result in increased fecal excretion of bile salts or exposure of the gastrointestinal tract to increased concentrations of secondary bile acids. In contrast, the presence of bile salt hydrolyzing bacteria appears to attenuate the increased production of "toxic" secondary bile acids associated with the consumption of a high-fat diet in pigs.

In contrast to the above studies, inconclusive evidence on the effect of BSH active microorganisms on fecal excretion of bile acids is been presented by De Smet *et al.* (1998), who evaluated effects of BSH active *L. reuteri* on cholesterol lowering in Seghers hybrid (sow) x Pietrain (boar) pigs. Twenty (20) male (castrated) and female pigs (30 kg) were fed a high-fat, high-cholesterol, low-fiber diet for 10 weeks, and a regular diet for the last 3 weeks. The animals were divided into 2 groups with 1 group administered *L. reuteri* ($1.8x10^{11}$ CFU/day twice daily) for a 4-week period. In both groups of animals' consumption of the high-fat diet resulted in significant increases in serum total cholesterol, and LDL cholesterol levels. The rise in serum

cholesterol was significantly lower in the animals consuming the *L. reuteri* supplemented diets, an effect that was sustained throughout the treatment and 3-week post-treatment intervals. The authors also evaluated the effect of *L. reuteri* consumption on fecal total bile levels using the 3 α-hydroxysteroid dehydrogenase colorimetric assay. Using this method significant increases in fecal total bile acids were observed (~40%) in the treatment animals relative to controls. However, when qualitative gas chromatographic analyses of total fecal bile acids, and secondary bile acids were conducted no between group differences were noted. The reason(s) for these discrepant findings is unclear, and as discussed, the use of colorimetric based analyses methods for quantitation of bile acids may not be suitable for experimental studies in animals administered large doses of microorganisms in conjunction with a high-cholesterol diet.

Park *et al.* (2008) evaluated the effects of *Lactobacillus acidophilus* and a mixture of *Lactobacillus casei* (*L. casei*) and *Bifidobacterium longum* (*B. longum*) on serum cholesterol levels and fecal sterol excretion in hypercholesterolemic pigs. Sixty (60) cross-bred (Landrace x Yorkshire x Duroc) male pigs (50.1±1.5 kg) were randomized to 1 of 2 groups (phase I) administered a normal diet (control group; n=10), or a hypercholesterolemic diet (n=50) for a period of 15 days. At the end of phase I, animals within the control group were maintained on the normal diet for an additional 20 days, and the animals consuming the hypercholesterolemic diets were randomized into 1 of 3 dietary intervention groups: 1) hypercholesterolemic control diet; 2) hypercholesterolemic diet supplemented with *L. acidophilus* ATCC 43121 ($3x10^7$ CFU/pig); 3) or a hypercholesterolemic diet supplemented with a mixture of *L. casei* and *B. longum* ($3x10^7$ CFU/pig strains not reported). Phase II diets were consumed daily for 20 days. At the end of the treatment period concentration of fecal sterols was evaluated using gas liquid chromatography. The authors reported statistically significant increases in the secondary bile acids deoxycholic acid and lithocholic acid in the animals consuming the microbial supplemented diets (Table IV.I-5). Similar to the previous study conducted by the authors, the quantitative distribution of bile acids suggests that the analytical methods used in the experiment may be inappropriate for analyses of bile acids in pigs. The major primary bile acids produced in pigs are hyocholic acid, and chenodeoxycholic acid, and their corresponding secondary bile acids are hyodeoxycholic acid and lithocholic acid respectively (Elliot, 1985). No quantitative measurements of hyocholic acid or hyodeoxycholic acid were presented by the authors indicating that the bile acid data is incomplete. In addition, the authors report that the concentrations of deoxycholic acid far exceed those of lithocholic acid; quantitatively this would not be expected since the primary bile acid cholic acid is not a major bile acid in pigs, and this finding strongly suggests that the analytical method employed by the authors is in either in error or inappropriate for the conditions of the experiment. The significance of the authors' findings therefore could not be determined.

Table IV.I-5 Fecal Bile Acid Excretion of Pigs at End of Phase II

Bile Acid	ND	HCD	*Lactobacillus acidophilus*		Mixture of *Lactobacillus casei* and *Bifidobacterium longum*	
			10 days	20 days	10 days	20 days
CA	1.4±0.2	1.5±0.1	1.3±0.2	1.5±0.1	1.5±0.1	1.6±0.1
CDCA	0.8±0.1	0.9±0.1	0.7±0.1	0.8±0.1	1.0±0.2	1.0±0.2
DCA	10.2±1.2[b]	10.8±1.8[b]	15.8±1.1[a]	16.5±1.9[a]	13.9±1.4[a]	14.5±1.6[a]
LCA	4.1±0.5[c]	6.0±0.6[b]	9.3±1.1[a]	10.0±1.4[a]	8.66±1.4[a]	9.9±1.3[a]

CA = cholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; LCA = lithocholic acid; ND = normal diet; HCD = hypercholesterolemic diet. Each value is the mean ± standard deviation. Means with different superscript within a row are significantly different by Duncan's multiple-range test ($p<0.05$)
Adapted from Park *et al.* (2008)

The ability of bile acids to precipitate in the presence of calcium salts is well established (Gu *et al.*, 1992; Govers *et al.*, 1994). In a study by De Rodas *et al.* (1996), the combined effect of dietary calcium and the bile salt active microorganism *Lactobacillus acidophilus* ATCC 43121 (*L. acidophilus*) was evaluated using 33 Yorkshire barrows (~92 kg sex not reported). Following a 14-day hypercholesterolemic run-in period, the animals were removed from their high cholesterol diets and randomized to 1 of 4 diets containing 0.7 or 1.4% dietary calcium, with or without, *L. acidophilus* ($2.5x10^{11}$ cells per feeding). Serum total cholesterol and total bile acids status was evaluated every 2 days *via* surgically installed catheters throughout the 15-day treatment period. Bile acid analysis method was not reported. The authors reported that serum cholesterol and bile salt concentrations were decreased throughout the treatment periods in all groups, effects that were dose responsive for calcium. In the animals randomized to the calcium + *L. acidophilus* treatment group, total serum bile acids were reduced by 23.9% ($P=0.061$) relative to the animals in the calcium supplement diets. The reduction in cholesterol concentrations observed in the study are assumed to be a result of hepatic synthesis of bile acids (from cholesterol) to replace those lost in the feces as calcium bile precipitates. The results of this study not only confirm that that dietary calcium can significantly impair the bioavailability of digestive bile salts, but indicates that studies in which cholesterol lowering microorganisms are administered in the diet within feed matrices that are rich in calcium (yogurt, milk), should be interpreted with the understanding that the purported effects of bile salt active microorganism on bile salt homeostasis may be influenced in part, or in whole, by the presence of calcium within the diet.

Administration of Bile Salt Active Microorganisms to Humans

The effects of *L. reuteri* NCIMB 30242 on fecal bile acid excretion were evaluated by Micropharma as secondary endpoints within the clinical trial described in Section IV.D (Jones *et al.*, 2011). In brief, the study was conducted using a double-blinded, multi-center, placebo-controlled, randomized, parallel-arm design. The study lasted a total of 10 weeks including 2-week wash-out, 2-week run-in, and 6-week treatment period. Three hundred seventy-five (375) subjects were screened, of which 159 were enrolled, 120 were randomized, 114

completed the study ITT, and 109 completed the study *per* protocol (PP). Subjects were randomly assigned to 2 groups: yogurts containing microencapsulated *L. reuteri* NCIMB 30242 or placebo yogurts. Measurements of total fecal bile acid concentrations were conducted on lyophilized fecal samples obtained from 19 placebo and 21 treatment subjects that were randomized to the sub-population evaluated for safety endpoints. Fecal samples were analyzed as *n*-butyl ester-trimethylsilyl ether derivatives, without prior isolation from the stool using validated gas chromatographic methods as described by Batta *et al.* (2002). .

As discussed previously, and presented in detail in the following sections (Table IV.I-8), significant reductions in total cholesterol were observed between the treatment and placebo group, an effect supporting that *L. reuteri* NCIMB 30242 effectively colonized the small intestine. In subjects randomized to the *L. reuteri* groups, a 14.4% reduction in total fecal bile acids was observed, however this effect was not statistically significant. This observation is consistent with the expected short-circuiting of enterohepatic circulation that would occur following the introduction to, and temporary residence of BSH active microorganisms within the small intestine.

Table IV.I-6 Effect of 6 Weeks of *Lactobacillus reuteri* Consumption on Total Fecal Bile Acids

Treatment	Baseline	Week 6	% Difference
Placebo (n=19)	3.08 (0.56)	3.26 (0.55)	5.8
L. reuteri NCIMB (n=21)	3.33 (0.48)	2.85 (0.43)	-14.4

Adapted from Jones *et al.* (2011). Data expressed as mean followed by standard error of mean in parentheses.

A comprehensive search of the literature was conducted to identify studies in which BSH active microorganism were administered to subjects and monitored for effects on fecal bile acid homeostasis as primary or secondary endpoints. Two studies were indentified.

Marteau *et al.* (2002) conducted a placebo controlled double-blind cross-over study using 36 women administered 1 of 2 yogurt treatments (control or *B. animalis* containing yogurt) over 4 consecutive 10-day periods. As discussed previously, *B. animals* DN-173 010 is a BSH active microorganism. During periods 2 and 4, subjects consumed three 125 g servings of yogurt each containing $1x10^8$ CFU of *B. animals* DN-173 010, or a control yogurt without Bifidobacteria. Periods 1 and 3 were represented by 10-day run-in and washout periods respectively. Total, primary and secondary bile acid concentrations of the feces were evaluated after each consumption period using gas chromatography and mass spectrometry as described by Setchell *et al.* (1983). The author's concluded that "*B. animalis* ingestion did not influence the fecal excretion of secondary bile salts."

A recent study by Ooi *et al.* (2010) evaluated the effect of oral consumption of capsules containing *L. acidophilus* CHO-220 and inulin on cholesterol metabolism. Thirty-two (32) hypercholesterolemic (cholesterol concentration ≥5.20 to ≤6.20 mmol/L, and LDL level of

≤4.20 mmol/L), yet otherwise healthy subjects were randomized to 1 of 2 groups (n=16 per group) administered capsules containing a combination of *L. acidophilus* CHO-220 ($1x10^9$ CFU/capsule) and inulin (0.20 g) or rice starch (placebo control). Each subject was instructed to consume 4 capsules daily for a duration of 3 months. At Weeks 0, 6, and 12, measurements of plasma bile acids (cholic acid, lithocholic acid, chenodeoxycholic acid, and deoxycholic acid) were obtained using gas chromatographic methods. Despite a significant reduction (-10%; P<0.05) in plasma total cholesterol and LDL cholesterol in the *L. acidophilus* group relative to controls, the authors reported no differences in plasma bile acid concentrations, indicating that the consumption of bile acid hydrolyzing microorganisms did not disrupt the enterohepatic circulation of bile acids (*via* excretion in the feces).

Summary

Based on concerns that repeated consumption of bile salt hydrolase active microorganisms in the diet may result in increased excretion of bile acids, a comprehensive search of the literature was conducted to identify studies evaluating bile acid physiology in animals and humans; studies investigating the effect of repeated consumption of BSH microorganisms in rodents, pigs and humans also were obtained. Based on the available literature, Micropharma concluded that there is no physiological basis for the assumption that deconjugation of bile acids proximal to the terminal ileum would prevent their re-absorption and result in increased loads of bile acids in colon. Evidence that consumption of high fat diets supplemented with BSH active microorganisms results in increased fecal concentrations of bile acids is suggested by a number of studies in rodents. However, these studies are confounded by incomplete analyses of bile acid content, and the use of non-validated, non-qualitative analyses of bile acids complicate interpretation of the results. In contrast, studies conducted in humans and pigs involved the use of gas chromatographic analysis, and use of internal standards for quantification of bile acids. Consistent with the expected capacity of deconjugated bile acids to be readily absorbed in the small intestine, these studies show that consumption of BSH active microorganism does not result in increased fecal loads of bile acids. Similarly, direct evidence obtained from the consumption of *L. reuteri* NCIMB 30242 in hypercholesterolemic volunteers over a 6-week repeat dose consumption period also did not result in increased fecal concentration of bile acids.

(iii) Effects of Bile Salt Active Microorganisms on Cholesterol Metabolism

L. reuteri NCIMB 30242 was selected for its BSH activity. The consumption of BSH active microorganisms has been shown to reduce serum cholesterol concentrations in hypercholesterolemic subjects. The mechanism for this effect is unclear, and several hypotheses have been suggested: Conjugated bile acids are important regulators of cholesterol homeostasis *via* their capacity to facilitate mixed micelle formation. Significant pre-ileal deconjugation of bile salts may impair cholesterol absorption by reducing the efficiency by which cholesterol is incorporated into mixed micelles. Alternatively, increased fecal excretion of

deconjugated bile acids also has been suggested; because bile is synthesized in the liver using cholesterol as a the sterol substrate, a net reduction in bile acid re-absorption *via* processes that interfere with the enterohepatic circulation of bile acids, results in the necessity of the liver to utilize endogenous cholesterol for the synthesis and replacement of lost stores, thereby reducing the circulating concentrations of cholesterol. As discussed, this latter mechanism of impaired enterohepatic circulation is inconsistent our current understanding of gastrointestinal physiology, as the production of deconjugated bile acids are readily absorbed in the neutral pH of the small intestine. *In vitro* studies have suggested that in the presence of high cholesterol and bile salt concentrations, high BSH activity may result in the formation of a cholesterol-bile acid complex which then precipitates out of solution, sequesters to the surface of the bacteria, or is assimilated by the organism (Gilliland *et al.*, 1985; Klaver *et al.*, 1993; Marshall and Taylor, 1995; Tahri *et al.*, 1996, 1997; Brashears *et al.*, 1998; Dambekodi and Gilliland, 1998; Pereira *et al.*, 2003). These purported mechanisms remain to be confirmed under *in vivo* settings; bile salts excreted in the feces *via* these mechanisms would not be expected to be bioavailable for dehydroxylation to secondary bile acids by the gut microflora in the colon. Recent studies have identified a novel mechanism by which deconjugation of bile acids may affect cholesterol status. Johnson *et al.* (2010) reported that through interactions with lipids and cholesterol, deconjugated bile acids can effectively stabilize the adenosine triphosphate binding cassette (ABC) cholesterol efflux transporter leading to a reductions in the absorption efficiency of dietary cholesterol and potentially increased excretion of circulating cholesterol into the bile, both processes leading to a net loss of cholesterol into the feces. Evidence supporting this mechanism of action in the cholesterol reduction observed through consumption of BSH active microorganism has yet to be proven.

Several studies were identified in the literature in which the administration of BSH active microorganisms to animals or humans results in nutritionally beneficial changes to cholesterol homeostasis in hypercholesterolemic individuals; studies conducted in normocholesterolemic animals or humans are limited or absent. Although a large number of studies characterizing the effects of various microorganisms and fermented milk products on cholesterol metabolism have been published in the public domain, only studies in which the strain was identified and characterized as a bile salt hydrolase active microorganisms were considered during the review. Additionally, since there are qualitative and quantitative limits to extrapolation of findings on cholesterol metabolism in animals to humans, studies conducted in animals are presented in brief, and a focus of the literature review was therefore placed on the identification of clinical data.

Studies Conducted with BSH Active Microorganisms in Animals

Lactobacillus reuteri NCIMB 30242

Lactobacillus reuteri NCIMB 30242 (*L. reuteri* NCIMB 30242) was evaluated for safety and cholesterol lowering efficacy in 33 BioF1B hamsters (7 to 8 weeks of age) (Micropharma, 2009).

The hamsters were fed diets containing 10% total fat and 0.05% cholesterol for 5 weeks to induce hypercholesterolemia. At the end of the hypercholesterolemic run-in period 22 animals were randomized to 1 of 2 treatment groups administered sterile saline (control) or microencapsulated *L. reuteri* NCIMB 30242 *via* gavage as 1.5 g capsules. Weight gain, feed consumption and feed conversion efficiency were monitored weekly. At baseline and Week 6, blood samples were taken to measure standard parameters related to safety: alanine aminotransferase, aspartate aminotransferase, lipase, urea, creatinine, glucose, calcium, phosphate, C-reactive protein, sodium, chloride, potassium, and bicarbonate. Hematological parameters were measured at Week 6 of treatment and included, white blood cells, red blood cells, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, neutrophils, lymphocytes, monocytes, eosinophils, large unstained cells, basophils, and reticulocytes. Cholesterol metabolism was evaluated *via* measures of TC, HDL cholesterol, and triglycerides (TG) at pre-induction baseline, after 4 and 5 weeks of induction and Weeks 2, 4, and 6 of treatment. LDL cholesterol was calculated according to the Friedewald formula ([LDL] = TC-HDL-(TG/2.2).

No significant differences in body weights, food intake or the food efficiency ratio were observed between the control and treatment groups. Likewise, no statistically significant differences were noted in any of the blood safety indices measured or standard hematological parameters.

Hypercholesterolemia was induced in all animals by the end of the induction phase (57.4% increase in total cholesterol). Six (6) hamsters per group were considered hypercholesterolemic responders and had increases in total cholesterol of 83.5%. Following treatment with *L. reuteri*, no statistically significant reduction in total cholesterol or LDL cholesterol was observed compared to control animals; however, a statistically significant reduction from baseline was reported (-35.2%; P=0.007 and -51.2%; P=0.007 for total cholesterol and LDL cholesterol, respectively). Among the hypercholesterolemic responders, animals randomized to the *L. reuteri* group had more pronounced reductions in total cholesterol (-41.2%; P=0.001) and LDL cholesterol (-56.5%; P=0.002) relative to baseline. HDL-cholesterol was significantly reduced (19.8%; P=0.001) compared to baseline in the *L. reuteri* treated animals. Reductions in control animals of total cholesterol, LDL and HDL cholesterol were not statistically significant. Although trends towards reduction in triglycerides were reported in treated animals, compared to baseline, significance was not reached at the conclusion of the study.

Findings Reported with Other Strains

Several additional studies were identified in the literature, which demonstrate that repeated oral administration of BSH active microbes to hypercholesterolemic animals results in beneficial nutritional changes in cholesterol metabolism. An overview of each study is provided in Table IV.I-7. As noted in the table below, effects on cholesterol metabolism have been reported in multiple species (hamsters, rats, mice, and pigs) administered various species and strains of lactic acid microorganisms in the diet. Typical doses of microorganisms administered in the diet

are between $1x10^8$ to $1x10^{11}$ CFU/animal/day. Biochemical changes in cholesterol metabolism vary dramatically between studies, an observation that is likely the result of differences in study design, animal species/strain, test article species/strain, dose, and matrix effects *etc.*

Table IV.I-7 Animal Studies Evaluating the Effect of Dietary Administration of BSH Active Microorganisms on Cholesterol Metabolism

Target Species	Microorganism	Dose and Duration of Treatment	Key Outcomes	Reference
Hamster				
BioF(1B) hamsters (n=36)	*Lactobacillus reuteri* NCIMB 30242 (microencapsulated)	0.12 g of test article/day (6 weeks)	Non-significant reduction in total cholesterol and trending towards decreased LDL cholesterol compared to control animals.	Micropharma, 2009
Male F(1)B hamsters (number not reported)	Microcapsulated *Lactobacillus fermentum* 11976	0 or 12.51 log CFU/mL twice daily by oral gavage (18 weeks).	Serum total cholesterol and LDL-cholesterol were significantly reduced (21.36 and 31.43%, respectively), compared to control animals. Serum triglycerides also reduced (significance not reported).	Bhathena *et al.*, 2009
Rat				
Sprague-Dawley rats (n=30)	*Lactobacillus plantarum* Lp91 (normal and microencapsulated) and *Lactobacillus plantarum* Lp21 (normal)	0 or $\geq 1x10^8$ CFU/g (21 days)	Reduction in total cholesterol (23.26, 15.71 and 15.01%), triacylglycerides (21.09, 18.77 and 18.17%), and LDL-cholesterol (38.13, 23.22 and 21.42%) with increased HDL-cholesterol (18.94, 10.30 and 7.78%) in Lp91, Lp91 (encapsulated) and Lp21, respectively (significance not reported). Fecal excretion of cholic acid increased in probiotic groups, compared to control group.	Kumar *et al.*, 2011
Rat (species/number not reported)	*Lactobacillus acidophilus* 4356	0 or $1x10^9$ CFU/day (duration not reported)	Significantly lower total serum cholesterol, LDL cholesterol and total liver cholesterol compared to control group.	Huang *et al.*, 2010
Rat (species/number not reported)	*Lactobacillus plantarum* MA2	$1x10^{11}$ cells/animal/day (duration not reported)	Significant reduction in serum total cholesterol, LDL-cholesterol and triglycerides. Liver total cholesterol and triglycerides also reduced. Fecal cholesterol and triglycerides significantly increased.	Wang *et al.*, 2009
Sprague-Dawley rats (n=24)	*Bifidobacterium longum* SPM1207	0.2 mL/day ($1x10^8$ to $1x10^9$ CFU/mL) (14 days)	Significant reduction in serum total cholesterol and LDL-cholesterol concentration. Also, increased fecal moisture content.	Lee *et al.*, 2009b

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Table IV.I-7 Animal Studies Evaluating the Effect of Dietary Administration of BSH Active Microorganisms on Cholesterol Metabolism

Target Species	Microorganism	Dose and Duration of Treatment	Key Outcomes	Reference
Sprague-Dawley rats (n=36)	*Lactobacillus acidophilus* ATCC 43121	$2x10^6$ CFU/day (21 days)	Probiotic treatment resulted in reduction of total serum cholesterol (25%) and VLDL+IDL+LDL cholesterol (42%) compared to control groups. Treated animals were reported to have a significant increase in fecal acid sterol excretion, and decrease in hepatic cholesterol 7α-hydroxylase mRNA expression. Also, decreased excretion of primary bile salts, cholic acid and chenodeoxycholic acid and increased excretion of secondary bile salts, deoxycholic acid and lithocholic acid.	Park *et al.*, 2007
Sprague-Dawley male rats (n=21)	*Bifodobacterium longum* BL1 *vs. Streptococcus thermophilus* + *Lactobacillus delbreuckii* subsp. *bulgaricus vs.* control	3.1 to $4.9x10^7$ CFU (BL1)/g diet (3 weeks)	Mean serum total cholesterol and triglycerides significantly reduced in BL1 group compared to the other 2 groups. Slight (non-significant) increase in fecal total bile acid concentration in BL1 group.	Xiao *et al.*, 2003
Rat (species/number not reported)	*Bifidobacteria breve* K110, *B. breve* K-111 and *B. infantis* K-525	0.5% in the diet (duration not reported)	K-110 and K-111 significantly diminished the increase of serum total cholesterol and LDL cholesterol compared to control (with K-111, reduced by 57 and 55%, respectively) compared to control group. Significant acceleration of normalizing activity of serum cholesterol in treated rats.	Rhee *et al.*, 2002
Rat (species/number not reported)	*Lactobacillus gasseri* SBT0270	Effective dose: $1x10^9$ CFU/day (duration not reported)	Reported hypocholesterolemic effect of treatment was attributed to suppression of bile acid reabsorption and enhanced excretion of acidic steroids.	Usman and Hosono, 2001
Rat (species/number not reported)	*Lactobacillus gasseri* SBT0270	Dose/duration not reported. Fed *via* fermented and non-fermented milk.	Significant reduction in serum total cholesterol, LDL cholesterol and bile acids.	Usman and Hosono, 2000

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Table IV.I-7 Animal Studies Evaluating the Effect of Dietary Administration of BSH Active Microorganisms on Cholesterol Metabolism

Target Species	Microorganism	Dose and Duration of Treatment	Key Outcomes	Reference
Mice				
ICR Mice (number not reported)	*Lactobacillus fermentum* SM-7	Dose/duration not reported.	Significant reduction in serum total cholesterol, total triglyceride levels, and LDL-cholesterol concentrations, compared to control animals.	Pan *et al.*, 2011
Male C57BL/6 mice (number not reported)	*Lactobacillus plantarum* KCTC3928 (doubly-coated with proteins and polysaccharides)	Live and inactivated bacteria (dose/duration not reported)	LDL-cholesterol and plasma triacylglycerol levels significantly reduced by 42% and 32% respectively, while fecal bile acid secretion increased by 45%, compared to control. Also, CYP7A12 gene expression and protein levels significantly up-regulated with live bacteria.	Jeun *et al.*, 2010
Mice (n=14) (species not reported)	*Lactobacillus collinoides* JCM1123(T)	(10 days of treatment (dose not reported)	Significantly lower total plasma cholesterol concentration, compared to control.	Tamura *et al.*, 2009
Mice (species/number not reported)	*Lactobacillus plantarum* PH04	0 or $1x10^7$ CFU/animal/ day (14 days)	Serum cholesterol and triglycerides were reduced by 7 and 10%, respectively, compared to control (significance not reported).	Nguyen *et al.*, 2007
Swiss Albino mice (n=30)	*Lactobacillus reuteri* CRL 1098	0 or $1x10^4$ cells/day (7 days)	Treatment resulted in significant increase in HDL to LDL ratio (by 17%), decrease in total cholesterol (20%) and triglycerides (33%), compared to the control group.	Taranto *et al.*, 2000
Swiss Albino mice (n=20)	*Lactobacillus reuteri* CRL 1098	0 or $1x10^4$ cells/day (7 days)	Significantly decreased total cholesterol (by 38%) and triglycerides (by 40%). Also, 20% increase in HDL to LDL ratio (significant). No translocation of native microflora into spleen or liver.	Taranto *et al.*, 1998
Mice (species/number not reported)	Combination of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*	0, 0.01 or 3.0% (v/v) in yogurt (56 days)	Mean serum cholesterol and LDL cholesterol significantly decreased. No effect on HDL cholesterol.	Akalin *et al.*, 1997

Table IV.I-7 Animal Studies Evaluating the Effect of Dietary Administration of BSH Active Microorganisms on Cholesterol Metabolism

Target Species	Microorganism	Dose and Duration of Treatment	Key Outcomes	Reference
Pigs				
Male cross-bred pigs (n=60)	*Lactobacillus acidophilus* 43121 and *Lactobacillus casei* + *Bifidobacterium longum*	$3x10^7$ CFU/day (10 or 20 days)	Serum total cholesterol and liver cholesterol concentrations significantly reduced in both treatment groups, compared to control group. Treatments also increased HDL-cholesterol to total cholesterol ratio and decreased serum bile acid concentration. Fecal cholesterol excretion increased by mixture of bacteria, but not so with *L. acidophilus*.	Park *et al.*, 2008
Large White pigs (n=12)	*Bifidobacterium animalis* DN-173 010	$3.5x10^{11}$ CFU, twice daily of living and inactivated bacteria [intraduodenally] (2 weeks)	Unconjugated bile acids made up 44 and 53% of total bile acids after 1 and 2 weeks of treatment compared to 25% before treatment or treatment with inactivated bacteria.	Lepercq *et al.*, 2004
Seghers hybrid x Pietrain pigs (n=20)	*Lactobacillus reuteri*	11.25 log10 CFU/day (4 weeks)	Significant reduction in total and LDL-cholesterol (no effect on HDL-cholesterol) compared to control animals. Significantly increased fecal bile salt excretion was also observed, compared to controls.	De Smet *et al.*, 1998
Göttingen male minipigs (n=6)	*Lactobacillus johnsonii* BFE 1059 and BFE 1061, *Lactobacillus reuteri* BFE 1058	$2x10^{12}$ CFU/day (5 weeks) (no control group)	All treatments lowered serum cholesterol (significance not reported) and increased triglyceride concentrations (non-significantly; possibly due to duration of feeding of high-cholesterol diet to obtain hypercholesterolemic state, *i.e.* 17 weeks). Also, probiotic treatment resulted in a definitive increase in fecal moisture content.	du Toit *et al.*, 1998
Yorkshire barrows (n=33)	*Lactobacillus acidophilus* ATCC 43121 with 0.7 or 1.4% calcium	$2.5x10^{11}$ cells/feeding (15 days)	Significantly reduced total cholesterol (by 11.8%), non-significant reduction in total serum bile acid concentration, compared to control animals.	De Rodas *et al.*, 1996
Boars (n=18)	*Lactobacillus acidophilus*	0.454 kg/(hd.d) yogurt (duration not reported)	Significant reduction of serum cholesterol and non-significant reduction in LDL-cholesterol, compared to control animals.	Danielson *et al.*, 1989

CFU = colony forming units; HDL = high-density lipoprotein; LDL = low-density lipoprotein; VLDL = very low density lipoprotein

Effect of BSH Active Microorganisms on Cholesterol Metabolism in Humans

The effect of repeated consumption of yogurt supplemented with *L. reuteri* is reported by Jones *et al.* (2011). As described in detail in Section 5.3.1, the study was designed according to a standard double-blinded placebo-controlled randomized parallel-arm multi-center design lasting a total of 10 weeks. This included a 2-week wash-out period, a 2-week run-in period in which subjects consumed placebo yogurts twice daily at breakfast or dinner, and a 6-week treatment period in which subjects consumed either placebo or treatment yogurts twice daily at breakfast or dinner. Subjects met with the investigational team at 5 different time points – Visit V0 (Week -4), V1 (Week -2), V2 (Week 0, randomization and treatment baseline), V3 (Week 3, treatment midpoint) and V4 (Week 6, treatment endpoint). Dietary intake, including information on total kcal, % fat, % carbohydrates and % protein, for subjects consuming placebo yogurts and treatment yogurts was measured at baseline (Week 0) and endpoint (Week 6) of the treatment period. Blood for assessment of lipid profile was collected at each visit (Table IV.I-8). Serum samples were analyzed enzymatically for LDL-C (primary efficacy variable), TC, HDL-C, TG, and ApoB-100.

The mean serum lipid concentrations at baseline and after 3- and 6-week consumption of placebo and treatment yogurts are shown in Table IV.I-8. After 3 weeks, there were non-significant decreases in the treatment group, as compared to control, of 4.06% (p=0.13) in LDL-C, 2.67% (p=0.26) in TC, and 3.77% (p=0.21) in apoB100. Also, a significant decrease in TG of 23.30% (p=0.024) was seen and there was a non-significant increase of 0.65% (p=0.86) in HDL-C at the 3 week time point. Over the 6-week treatment period, consumption of microencapsulated *L. reuteri* NCIMB 30242 significantly reduced the LDL-C concentration, from baseline as compared to placebo, by 8.92% (p=0.006) and 9.23% (p=0.006; data not shown) for ITT and PP populations respectively. Also over the 6-week treatment period, significant reductions of 4.81% (p=0.045) in TC, and of 6.81% (p=0.046) in apoB-100, from baseline as compared to placebo, were seen. A 19% increase in serum triglycerides relative to the placebo treatment was observed at week 6 relative to baseline; however this change was not statistically significant. No statistically significant between group changes in (p=0.982) in HDL-C was reported at the 6-week endpoint.

Table IV.I-8 Effect of *Lactobacillus reuteri* NCIMB 30242 Consumption on Measures of Cholesterol Metabolism

	Placebo	*L. reuteri* NCIMB 30242	Placebo	Treatment
	TC (mmol/L)		**LDL-C (mmol/L)**	
Start (Week 0)	6.65 (0.09)	6.68 (0.12)	4.23 (0.06)	4.37 (0.08)
Midpoint (Week 3)	6.47 (0.11)	6.26 (0.10)	4.28 (0.09)	4.20 (0.08)
Endpoint (Week 6)	6.23 (0.11)	5.91 (0.11)	4.27 (0.10)	4.00 (0.11)
Week 0 *vs.* Week 3				
Change	-0.18 (0.11)	-0.40 (0.13)	0.05 (0.08)	-0.15 (0.09)
Change (%)	-2.28 (1.55)	-4.95 (1.72)	1.49 (1.87)	-2.57 (1.84)
Change relative to control		*-2.67*		*-4.06*
Week 0 *vs.* Week 6				
Change	-0.42 (0.11)	-0.77 (0.13)	0.05 (0.08)	-0.37 (0.11)
Change (%)	-5.76 (1.58)	-10.57 (1.79)	1.38 (2.16)	-7.54 (2.38)
Change relative to control		*-4.81*[a]		*-8.92*[b]
	HDL-C (mmol/L)		**LDL-C/HDL-C**	
Start (Week 0)	1.48 (0.05)	1.42 (0.05)	2.86 (0.12)	3.24 (0.10)
Midpoint (Week 3)	1.43 (0.06)	1.39 (0.05)	3.27 (0.16)	3.23 (0.13)
Endpoint (Week 6)	1.42 (0.06)	1.33 (0.05)	3.34 (0.17)	3.24 (0.16)
Week 0 *vs.* Week 3				
Change	-0.05 (0.04)	-0.03 (0.03)	0.21 (0.13)	0.00 (0.08)
Change (%)	-1.73 (2.90)	-1.07 (2.08)	8.62 (4.28)	0.03 (2.17)
Change relative to control		*0.65*		*-8.59*
Week 0 *vs.* Week 6				
Change	-0.06 (0.04)	-0.09 (0.05)	0.28 (0.13)	0.00 (0.13)
Change (%)	-3.30 (2.73)	-3.20 (3.22)	10.51 (4.55)	0.92 (3.84)
Change relative to control		*0.10*		*-9.60*
	TG (mmol/L)		**apoB-100 (g/L)**	
Start (Week 0)	1.60 (0.11)	1.62 (0.10)	1.13 (0.02)	1.17 (0.02)
Midpoint (Week 3)	1.82 (0.14)	1.52 (0.09)	1.04 (0.02)	1.03 (0.02)
Endpoint (Week 6)	1.43 (0.10)	1.72 (0.11)	1.02 (0.03)	0.98 (0.02)
Week 0 *vs.* Week 3				
Change	0.22 (0.11)	-0.08 (0.07)	-0.10 (0.02)	-0.14 (0.03)
Change (%)	25.22 (8.88)	1.92 (4.78)	-7.12 (1.96)	-10.89 (2.21)
Change relative to control		*-23.30*[a]		*-3.77*
Week 0 *vs.* Week 6				
Change	-0.17 (0.10)	0.10 (0.11)	-0.11 (0.03)	-0.19 (0.03)
Change (%)	-0.40 (6.20)	18.80 (10.03)	-8.21 (2.43)	-15.02 (2.30)
Change relative to control		*19.20*		*-6.81*[a]

Data expressed as mean followed by standard error in parentheses.
[a] Comparison between treatment and placebo groups was performed ($p<0.05$)
[b] Comparison between treatment and placebo groups was performed ($p<0.01$)

A comprehensive search of the literature was conducted to identify other clinical studies in which BSH active microorganisms were administered to healthy subjects (Table IV.I-9). Although a large number of clinical studies were identified in which microorganisms or fermented milk products were administered to subjects for evaluation of effects on cholesterol metabolism, only 4 studies were identified in which the test organism was explicitly characterized by the authors as BSH active. Based on the multiple mechanisms by which microorganisms may affect cholesterol metabolism, studies in which the BSH phenotype of the organism were not reported, were not considered during the safety assessment. Generalization of the findings reported by the authors was not possible due to the limited number of studies identified and the significant differences in their study designs (treatment duration, dose, microorganism, subject inclusion criteria, control of fat intake, *etc.*). Nevertheless, typically both total cholesterol and LDL cholesterol are reduced in the BSH consuming subjects with reductions between 1 to 20% reported. Effects on HDL cholesterol levels were highly variable between studies. Similarly conflicting changes in triglycerides were reported among the studies.

Table IV.I-9 Clinical Studies Evaluating the Effect of BSH Active Lactic Acid Bacteria Consumption on Measures of Cholesterol Metabolism

Study	Design	Subjects	Treatment	Cardiovascular Health Markers % Change from Baseline			
				ΔTotal Chol.	Δ LDL Chol.	Δ HDL Chol.	Δ Trig.
***Jones et al.* (2011)** Cholesterol Lowering efficacy of microencapsulated BSH-active *Lactobacillus reuteri* NCIMB 30242 yogurt formulation in humans	RPC(II)	120 Adult ♂/♀ hypercholesterolemic	1. Placebo yogurt 2. Yogurt + *L. reuteri* NCIMB 30242 ($1x10^{10}$ CFU/day) 6 weeks	-5.76 -10.57*	+1.38 -7.54*	-3.30 -3.20	-0.40 +18.8
***Ooi et al.* (2010)** *Lactobacillus acidophilus* CHO-220 and inulin reduced plasma total cholesterol and low-density lipoprotein cholesterol *via* alteration of lipid transporters	RPC (II)	32 Adult ♂/♀ hypercholesterolemic	1. Placebo capsule 2. *L. acidophilus* CHO-220 ($4x10^9$ CFU/day) + Inulin (0.8 g/day) 12 weeks	+1.76 -7.84*#	+2.17 -9.27*#	0.0 -8.02	+1.52 +2.50
***Xiao et al.* (2003)** Effects of milk products fermented by *Bifidobacterium longum* on blood lipids in rats and healthy adult male volunteers	RPC (II)	36 healthy ♂ adults	1. Placebo yogurt 2. Yogurt + *B. longum* BL1 ($3x10^8$ CFU/day) 4 weeks	-0.3 -2.7	-1.7 -3.2	-4.8 -4.5	+2.1 +5.2
Ashar and Prajapati (2000) Verification of hypocholesterolemic effect of fermented milk on human subjects with different cholesterol levels	NCI	27 Adults with various cholesterol ranges 1. <2.0 g/L 2. 2.0 – 2.2 g/L 3. 2.2 – 2.5 g/L 4. >2.5 g/L	*L. acidophilus* V3 capsules 1. 2. 3. 4. 20 days	 0 0 -8.7 -14.7	 4.1 0 -13.3 -20.0	 2.2 19.6 6.5 5.6	 -33.3 0 -5.3 -13.3

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Table IV.I-9 Clinical Studies Evaluating the Effect of BSH Active Lactic Acid Bacteria Consumption on Measures of Cholesterol Metabolism

Study	Design	Subjects	Treatment	Cardiovascular Health Markers % Change from Baseline			
				ΔTotal Chol.	Δ LDL Chol.	Δ HDL Chol.	Δ Trig.
Anderson and Gilliland (1999) Effect of fermented milk (Yogurt) containing *Lactobacillus acidophilus* L1 on serum cholesterol in hypercholesterolemic humans	Study 1 NCI	29 Adult ♂/♀ hypercholesterolemic	1. Yogurt + *L. acidophilus* L1 ($2x10^9$ CFU/day) 2. Yogurt + *L. acidophilus* ATCC 43121 ($2x10^9$ CFU/day) 3 weeks	-2.4* -0.91	-2.6 -1.1	-3.9* -5.9*	-3.2 +16.5
	Study 2 RPC (II)	40 Adult ♂/♀ hypercholesterolemic	1. Placebo yogurt 2. Yogurt + *L. acidophilus* L1 ($2x10^9$ CFU/day) 4 weeks	+0.3 -3.2*#	-0.2 -4.1*#	+0.8 -3.2*	-0.5 -3.4
Mohan *et al.* (1990) Preliminary observations on effect of Lactobacillus sporogenes on serum lipid levels in hypercholesterolemic patients	NCI	Adult 15♂/2♀ hypercholesterolemic	*L. sporogenes* ($1.8x10^{7}$ CFU/day)	-31*	-35*	+7.3*	+8.9

NCI = Non Controlled Intervention; RPC(II) = Randomized Placebo Controlled Parallel; RPC(x) = Randomized Placebo Controlled Cross-Over
*Significant difference *vs.* baseline (P<0.05)
#Significant difference *vs.* control (P<0.05)

J. General Recognition of Safety

As described, numerous unqualified strains of *L. reuteri* have a long-history of safe use in the food industry as a fermentation culture for the production of sourdough breads. Various strains of *L. reuteri* also have a history of safe use in food and supplement probiotic products. In the United States, *L. reuteri* DSM 17938 is GRAS for use in multiple food categories at use levels of up to $1x10^8$ CFU per serving; cumulative exposures of between $1x10^9$ to $1x10^{10}$ CFU/person were estimated during the GRAS determination. This GRAS self-affirmation, conducted by Biogaia AG (Biogaia), was Notified to the FDA on May 2008 (U.S. FDA, 2008). Within the Notification, the totality of scientific data on the safety of *L. reuteri* DSM 17938 was reviewed, and included information obtained from other strains of *L. reuteri* including clinical studies, animal studies, and other studies and information available within the public domain generated using various non-related strains *L. reuteri*. Other than the requirement for evaluation of antibiotic resistance, no evidence was identified by the Notifier to suggest that the species *L. reuteri* is unsafe for use in food. The FDA has reviewed these food uses of *L. reuteri* DSM 17938, and the Agency stated that it "has no questions at this time regarding BioGaia's conclusion that *L. reuteri* strain DSM 17938 is GRAS under the intended conditions of use." (U.S. FDA, 2008).

In the European Union *L. reuteri* containing probiotic food and supplement products are currently on the marketplace. Due to the long-history of use of *L. reuteri* in food and supplement products the species is not defined as a novel food ingredient and is therefore not subject to novel food regulations. Although specific authorization, by the European Commission, for sale of *L. reuteri* in food and supplement products is not required, safe use of the species in food and supplement products has been reviewed by the EFSA. This safety review was conducted under the Qualified Presumption of Safety (QPS) process. This assessment process recognizes that many microorganisms have long-histories of safe use by the food industry, and is based on 4 essential pillars of information: established identity, body of knowledge, possible pathogenicity, and end use. Following a review of the current uses of *L. reuteri* in food and feed products, EFSA concluded that the current food and feed uses of *L. reuteri* do not present cause for safety concern, and QPS status was granted for the species (EFSA, 2007).

K. Summary

Micropharma proposes to market *Lactobacillus reuteri* NCIMB 30242 for use as an ingredient in multiple food categories (beverage and beverage bases, breakfast cereals, cheeses, dairy product analogs, fats and oils, frozen dairy desserts, grain products and pastas, milk products, processed fruits and fruit juices, soft candy , and sugar substitutes) at use levels of up to $1x10^{10}$ CFU per serving.

The organism has been characterized phenotypically using API 50 carbohydrate fermentation profile strip testing, and APIzym enzymatic analyses. The fermentation and enzymatic profile generated by NCIMB 30242 is consistent with that expected by *L. reuteri* and related species, and can be used as an early stage identification method for the organism. Recently the genome of the organism has been sequenced using shotgun sequencing methods. From the sequence assembly, 91% of the reads were fully assembled to yield 112 large contigs, which were derived from a total genome coverage of 40x. The size of the genome was estimated to be 1.78 Mb. The contig sequences were then submitted to a RAST server for annotation, and identification of the gene content of the organism. Using gene sequence data for the entire 16S gene, a search of the GenBank database was conducted for confirmation of the species identity. A concise alignment (≥99%) of the 16S RNA sequence of multiple *L. reuteri* strains was confirmed. NCIMB 30242 also was sent to BCCM/LMG for AFLP analyses and generation of a strain specific DNA fingerprint. Using dendrogram computer analysis of the AFLP banding profile for NCIMB 30242, and comparison of this fingerprint to a large database of *L. reuteri* strains including the *L. reuteri* type strain (LMG 9213T), further confirmed the identity of *L. reuteri* at the species level. Based on the above information it can be concluded that *L. reuteri* NCIMB 30242 is adequately characterized at both the species and strain level.

L. reuteri NCIMB 30242 is currently deposited in an international culture bank (NCIMB) under the strain identification number NCIMB 30242. This culture deposit is currently used as a master culture for preparation of all commercial Micropharma products. NCIMB 30242 is manufactured using traditional fermentation methods at Chr Hansen (Denmark) under cGMP, and using permitted and suitable fermentation processing-aids. Quality control methods are implemented throughout various stages of fermentation to ensure production of a pure culture absent contaminating pathogens. To improve viability of the organism following consumption, the lyophilized powder is then encapsulated with food grade materials (sodium alginate, polyethylene glycol 1500, and Epsilon-polylysine) at a second contract research facility (BRACE GmbH, Germany). Suitable food grade specifications also have been developed for this material ensuring the final food grade product is of high purity, and free of contaminating microbes and heavy metals.

L. reuteri NCIMB 30242 is proposed for use as a food ingredient in multiple food categories as described above at use levels of up to 3.25% ($1x10^{10}$ CFU/serving). Estimates for the intake of Micropharma's *L. reuteri* NCIMB 30242 ingredient were based on the proposed food-uses and use-levels in conjunction with food consumption data included in the 2003-2004 and 2005-2006 NHANES (CDC, 2006, 2009; USDA, 2009), which provides the most appropriate data for evaluating food use and food consumption patterns in the U.S. Under the conditions of intended use and on an all-user basis, the mean intake of *L. reuteri* NCIMB 30242 by the total U.S. population from all proposed food-uses was estimated to be $1.8x10^{10}$ CFU/ person/day. The heavy consumer (90^{th} percentile) all-user intake of *L. reuteri* NCIMB 30242 by the total U.S. population from all proposed food-uses was estimated to be $3.5x10^{10}$ CFU/person/ day. On an

individual population basis, the greatest mean all-user intake of *L. reuteri* NCIMB 30242 on an absolute basis was determined to occur in children and male teenagers at $2.3x10^{10}$ CFU/ person/day. Exposure to the encapsulation agents under the proposed uses of *L. reuteri* NCIMB 30242 also was estimated. Exposure to these ingredients under the proposed of *L. reuteri* NCIMB 30242 would result in limited dietary exposures, and would not appreciably change background exposures to these ingredients in the U.S. diet; the proposed use of these materials as encapsulation agents was therefore considered GRAS.

A large body of safety data was reviewed during the safety assessment of *L. reuteri* NCIMB 30242, including both strain specific information and relevant safety data generated using related strains of *L. reuteri*. Although the use of *L. reuteri* for its nutritional value is a recent development, strains of *L. reuteri* have a long-history of safe use in the food supply; *L. reuteri* is commonly employed by the food industry for its fermentation properties, and specifically, is one of the most widely used microorganisms used during production of sourdough breads.

Information on the metabolic fate of orally administered *L. reuteri* is not available. However, given that the ingredient is a live microorganism, it is expected to transiently colonize the small intestine and colon following consumption, largely escaping digestive and absorptive processes. Organisms not surviving gastrointestinal transit would be metabolized by human digestive enzymes and the nutritive components of the cell (proteins, lipids, carbohydrates) would be used as a source of nutrients. Non-nutritive components would be further metabolized by the resident microflora of the colon, and/or excreted in the feces.

A comprehensive search of the literature did not identify toxicology studies conducted with *L. reuteri*. Strain specific toxicology studies conducted using *L. reuteri* NCIMB 30242 also have not been conducted. It is generally accepted that microbial-host interactions are species specific, and therefore the applicability of rodent toxicology studies to support the safety of probiotics intended for consumption by humans should be approached with caution since significant species difference between rodents and humans is likely to exist for microbial test products. Information supporting the history of use of a species and closely related species, the available body of evidence obtained through clinical investigations, and information on the antibiotic resistance and metabolic properties of the organism are typically sufficient to form the basis of safety for the majority of *Lactobacillus* species. This viewpoint is widely acknowledged in the literature.

The safety of NCIMB 30242 has recently been evaluated using a double-blind placebo controlled study design in 120 hypercholesterolemic male and female subjects. Encapsulated NCIMB 30242 was administered to treatment subjects *via* a yogurt matrix resulting in daily intakes of $5x10^{10}$ CFU/day. Subjects consumed the test yogurts on a daily basis over a period of 6 weeks. Measurements of cholesterol and fecal microflora and standard clinical chemistry and hematology safety endpoints were obtained at baseline and upon completion of the treatment period. No adverse events were reported, and no significant between group

differences in safety parameters was reported between the placebo and treatment groups. Consistent with the bile salt active phenotype of the organism, a statistically significant between group reduction in serum cholesterol levels from baseline was observed in the treatment subjects. This effect was <10%, and therefore was considered a beneficial nutritional response, particularly given the hypercholesterolemic status of the participants.

A large number of clinical studies conducted with various strains of *L. reuteri* were identified in the literature. In total 59 studies were obtained and reviewed for information relevant to the safety of *L. reuteri* consumption. These studies included trials conducted in healthy adults and children, and in groups of adults and children with various disease states or adverse physiological conditions requiring medical intervention (*e.g.*, atopic dermatitis, acute diarrhea, rotavirus infection). Within these studies *L. reuteri* has been administered at doses of up to $1x10^{11}$ CFU/person/day, and doses of between $1x10^{8}$ and $1.2x10^{9}$ CFU/person/day have been safely administered and well tolerated by children for durations of up to 1 year. Consumption of *L. reuteri* in these studies typically results in transient colonization of the colon, with colonization rapidly diminishing following discontinuation of probiotic administration. To date, no adverse findings attributable to *L. reuteri* have been reported in the literature, and based on this substantiate body of data conducted with numerous strains of *L. reuteri*, it can be generally concluded that the species is non-pathogenic, and non-toxigenic.

Lactobacillus reuteri NCIMB 30242 was specifically selected for its high bile salt hydrolase activity, and corresponding capacity to reduce cholesterol levels in animals and humans during repeated consumption of the organism. Numerous probiotic safety assessments and some regulatory Agencies have cautioned against the use of bile salt active microorganisms as food ingredients. The basis for this concern relates to the possibility that transient persistence of the microbes within the small intestine, and active deconjugation of bile acids proximal to the terminal ileum may result in increased colonic concentrations of bile acids, and a corresponding increased local and systemic exposure to "toxic" secondary bile acids, which may be undesirable. Micropharma noted that these concerns are largely hypothesis based, and that comprehensive reviews of the subject matter are not available within the public domain. The viewpoint that loss of loss of deconjugated bile acids in the feces is based exclusively on the belief that deconjugation of bile acids proximal to the terminal ileum reduces their solubility and prevents active transport/absorption of the bile acids in the small intestine. Corresponding increases in colonic concentrations of bile acids would then be subject to secondary microbial biotransformation events, and production of "toxic" secondary bile acids. A critical review of the literature for studies characterizing the metabolic fate and gastrointestinal physiology of bile acids in humans and animals was conducted. Additional studies evaluating the effect of BSH active microorganism consumption in pigs, and humans also were evaluated for evidence of increased fecal and systemic exposure to secondary bile acids when these organisms are consumed on a regular basis at high dietary levels. Based on a critical review of this information it was determined that widespread concerns related to loss of deconjugated bile

salts in the feces associated with deconjugation of bile acids proximal to the terminal ileum are not adequately supported by references to the primary literature, and are inconsistent with our comprehensive understanding of gastrointestinal physiology and the metabolic fate of bile acids. Moreover, a review of several studies conducted in pigs and humans administered bile acid active microorganisms in the diet on a repeat basis provide no collective evidence that consumption of BSH active microbes results in increased local and systemic exposures to secondary bile acids. It was therefore concluded that under the proposed uses of Micropharma's *L. reuteri* NCIMB 30242 in food that no increased risk of exposure to "toxic" secondary bile acids would occur. General recognition of this conclusion was supported by the E.U. PROSAFE Panel who considered the safety of bile salt hydrolase activity expression by microorganisms during their workshop on probiotic safety. The PROSAFE Panel concluded "that bile salt deconjugation activity is irrelevant for safety assessment and is not recommended for in vitro safety testing of probiotics." (Vankerckhoven *et al.*, 2008).

Consistent with the BSH active phenotype of *L. reuteri* NCIMB 30242, regular consumption of the microorganisms is associated with cholesterol reduction properties when administered to rodents and hypercholesterolemic humans. The mechanism for this effect is unclear, and several possibilities including disruption of bile acid enterohepatic circulation, and impairments in cholesterol absorption through reduced efficiency of mixed micelle formation have been suggested. Recent studies have shown that through interactions with lipids and cholesterol, deconjugated bile acids can effectively stabilize the adenosine triphosphate binding cassette (ABC) cholesterol efflux transporter leading to a reductions in the absorption efficiency of dietary cholesterol and potentially increased excretion of circulating cholesterol into the bile, both process leading to a net loss of cholesterol into the feces.

The antibiotic resistance profile of a microorganism is a strain specific phenotype, and *in vitro* antibiotic resistance testing has been conducted for NCIMB 30242 against a broad range of clinically important antibiotics. Antibiotic resistance profiles deviating outside the EFSA breakpoint values established for *L. reuteri* were only observed against chloramphenicol. Although these apparent deviations in the MIC values relative to the breakpoint MIC value of 4 µg/mL was slight and not consistently observed, moderate resistance to chloramphenicol should be assumed. As a result of this observation, the NCIMB 30242 genome was carefully search for chloramphenicol resistance determinants. Matches of the gene sequence data to homologs of chloramphenicol resistance genes were limited to a multi-drug transporter that was 23% identical to the chloramphenicol transporter FloR. It was therefore determined that the apparent chloramphenicol resistance is due to conditional difference in chloramphenicol uptake, and not a result of an acquired and potentially transmissible antibiotic resistance gene. No safety concerns related to antibiotic resistance were noted.

Several additional metabolic phenotypes of the organism including the production of D-lactic acid, biogenic amines, antimicrobials, and deconjugation of bile acids were investigated.

NCIMB 30242 was shown to ferment lactose to a racemic mixture of D- and L-lactic acid in a ratio of 45:55% respectively. Although D-lactate production by the gut microflora has been reported to result in D-lactic acidosis in subjects with short bowel syndrome, this effect is limited to this pathological condition and always in association with antibiotic use and consumption of a high carbohydrate diet. Humans can actively metabolize D-lactose, and studies conducted in infants consuming strains *L. reuteri* that produce quantitatively comparable levels of D-lactic acid did not result in elevations in serum D-lactate concentrations outside the normal range. Safety concerns related to D-lactic acidosis resulting from the consumption of D-lactic acid producing microorganisms was determined not to be relevant to the intended use of NCIMB 30242.

The capacity of NCIMB 30242 to synthesize biogenic amines was investigated under culture conditions optimized for biogenic amine production. Both colorimetric and HPLC analyses were employed for detection of biogenic amines in the fermentation media. No colorimetric reactions were observed, and the absence of putrescine, cadaverine, histamine, and tyramine at a detection level of 1 mg/L was supported by HPLC analyses.

Strains of *L. reuteri* are known to produce various antimicrobial products including various bacteriocins, and a novel glycerol derived compound, reuterin. Neither reuterin, nor the production of bacteriocins were identified.

Finally, a bioinformatic search of the annotated gene sequence was conducted to identify any potentially undesirable phenotypic properties of the organism. Forty percent of the identified genes were classified as established sub-systems based on functional roles in metabolic pathways or class of protein. Sixteen genes classified as virulence genes were identified. Four genes were associated with iron scavenging mechanisms and 12 genes were classified in the antibiotic resistance and toxic compound sub-systems. The antibiotic resistance genes were determined to be common to lactobacilli and not associated with mobile elements. No genes involved in pathogen adhesion, invasion, toxin or superantigen production, or regulation of virulence were identified in the analysis.

L. Conclusion

The weight of the scientific evidence presented herein indicates that the intended uses of *Lactobacillus reuteri* NCIMB 30242, meeting appropriate food-grade specifications and manufactured in-line with cGMP, are safe and suitable. The data and information summarized in this report demonstrate that the intended uses of *Lactobacillus reuteri* NCIMB 30242 would be GRAS, based on scientific procedures.

REFERENCES

Abrahamsson T, Jakobsson T, Sinkiewicz G, Fredriksson M, Björkstén B (2005). Intestinal microbiota in infants supplemented with the probiotic bacterium Lactobacillus reuteri. J Pediatr Gastroenterol Nutr 40(5):692 [abstract PN 1–17].

Abrahamsson TR, Jakobsson T, Böttcher MF, Fredrikson M, Jenmalm MC, Bjorksten B et al. (2007). Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. J Allergy Clin Immunol 119(5):1174-1180.

Akalin AS, Gönç S, Düzel S (1997). Influence of yogurt and acidophilus yogurt on serum cholesterol levels in mice. J Dairy Sci 80(11):2721-2725.

Anderson W, Gilliland SE (1999). Effect of fermented milk (yogurt) containing Lactobacillus acidophilus L1 on serum cholesterol in hypercholesterolemic humans. J Am Coll Nutr 18(1):43-50.

Anukam K, Osazuwa E, Ahonkhai I, Ngwu M, Osemene G, Bruce A W et al. (2006). Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo controlled trial. Microbes Infect 8(6):1450-1454.

Anukam KC, Osazuwa EO, Osadolor HB, Bruce AW, Reid G (2008). Yogurt containing probiotic *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 helps resolve moderate diarrhea and increases cd4 count in HIV/AIDS patients. J Clin Gastroenterol 42(3):239-243.

Ashar MN, Prajapati JB (2000). Verification of hypocholesterolemic effect of fermented milk on human subjects with different cholesterol levels. Folia Microbiol (Praha) 45(3):263-268.

Asli G, Alsheikh A, Weizman Z (2003). Prevention of infectious diseases in day care infants. J Pediatr Gastroenterol Nutr 36:530 [abstract 0041].

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA et al. (2008). The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9:75 [doi:10.1186/1471-2164-9-75].

Baijal PK, Fitzpatrick DW, Bird RP (1998). Comparative effects of secondary bile acids, deoxycholic and lithocholic acids, on aberrant crypt foci growth in the postinitiation phases of colon carcinogenesis. Nutr Cancer 31(2):81-89.

Batta AK, Salen G, Batta P, Tint GS, Alberts DS, Earnest DL (2002). Simultaneous quantitation of fatty acids, sterols and bile acids in human stool by capillary gas-liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 775(2):153-161.

Becker K, Southwick K, Reardon J, Berg R, Maccormack JN (2001). Histamine poisoning associated with eating tuna burgers. JAMA 285(10):1327-1330.

Begley M, Hill C, Cormac G, Gahan M (2006). Bile salt hydrolase activity in probiotics. Appl Environ Microbiol 72(3):1729-1738.

Beilke LD, Besselsen DG, Cheng Q, Kulkarni S, Slitt AL, Cherrington NJ (2008). Minimal role of hepatic transporters in the hepatoprotection against LCA-induced intrahepatic cholestasis. Toxicol Sci 102(1):196-204.

Bernardeau M, Guguen M, Vernoux JP (2006). Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiol Rev 30(4):487-513.

Bernardeau M, Vernoux JP, Henri-Dubernet S, Guéguen M (2008). Safety assessment of dairy microorganisms: the *Lactobacillus* genus. Int J Food Microbiol 126(3):278-285.

Bernstein H, Bernstein C, Payne CM, Dvorak K (2009). Bile acids as endogenous etiologic agents in gastrointestinal cancer. World J Gastroenterol 15(27):3329-3340.

Betta P, Sciacca P, Trovato L, Vilardi L, Scuderi MG, Di Benedetto V et al. (2007). Probiotics in the prevention of bacterial and *Candida* infections in newborns submitted to greater surgical interventions and admitted in NICU-retrospective group controlled study. J Pediatr Gastroenterol Nutr 44:e127 [abstract PG4-13].

Bhathena J, Martoni C, Kulamarva A, Urbanska AM, Malhotra M, Prakash S (2009). Orally delivered microencapsulated live probiotic formulation lowers serum lipids in hypercholesterolemic hamsters. J Med Food 12(2):310-319.

Björkman P (1999). *Colonization of the Human Gastrointestinal Tract by the Lactic Acid Bacteria Lactobacillus Reuteri. M.Sc. Thesis*, Helsinki, Finland: University of Helsinki, Department of Food Technology [Finnish – English abstract only]. Information available at: http://www.biogaia.com/fr/node/1587.

Böttcher MF, Abrahamsson TR, Fredriksson M, Jakobsson T, Bjorksten B (2008). Low breast milk TGF-β2 is induced by *Lactobacillus reuteri* supplementation and associates with reduced risk of sensitization during infancy. Pediatr Allergy Immunol 19(6):497-504.

Bover-Cid S, Holzapfel WH (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. Int J Food Microbiol 53(1):33-41.

Boyle RJ, Robins-Browne RM, Tang ML (2006). Probiotic use in clinical practice: What are the risks? Am J Clin Nutr 83(6):1256-1264.

Branton WB, Jones ML, Tomaro-Duchesneau C, Martoni CJ, Prakash S (2010). In vitro characterization and safety of the probiotic strain Lactobacillus reuteri Cardioviva NCIMB 30242. Int J Probiotics Prebiotics [Manuscript - June 25, 2010].

Brashears MM, Gilliland SE, Buck LM (1998). Bile salt deconjugation and cholesterol removal from media by Lactobacillus casei. J Dairy Sci 81(8):2103-2110.

Bruni FM, Piacentini GL, Peroni DG, Bodini A, Fasoli E, Boner AL (2009). Cow's milk allergic children can present sensitisation to probiotics. Acta Paediatr 98(2):321-323.

Cadieux P, Wind A, Sommer P, Schaefer L, Crowley K, Britton RA et al. (2008). Evaluation of reuterin production in urogenital probiotic *Lactobacillus reuteri* RC-14. Appl Environ Microbiol 74(15):4645-4649.

Caglar E, Cildir SK, Ergeneli S, Sandalli N, Twetman S (2006). Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium *Lactobacillus reuteri* ATCC 55730 by straws or tablets. Acta Odontol Scand 64(5):314-318.

Caglar E, Kavaloglu SC, Kuscu OO, Sandalli N, Holgerson P L, Twetman S (2007). Effect of chewing gums containing xylitol or probiotic bacteria on salivary mutans streptococci and lactobacilli. Clin Oral Investig 11(4):425-429.

Caglar E, Kuscu OO, Cildir SK, Kuvvetli SS, Sandalli N (2008). A probiotic lozenge administered medical device and its effect on salivary mutans streptococci and lactobacilli. Int J Paediatr Dent 18(1):35-39.

CDC (2006). *Analytical and Reporting Guidelines: The National Health and Nutrition Examination Survey (NHANES).* Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: http://www.cdc.gov/nchs/data/nhanes/nhanes_03_04/nhanes_analytic_guidelines_dec_2005.pdf.

CDC (2009). *National Health and Nutrition Examination Survey (NHANES): 2005-2006.* Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: http://www.cdc.gov/nchs/nhanes/nhanes2005-2006/nhanes05_06.htm.

Cheah PY, Bernstein H (1990). Colon cancer and dietary fiber: Cellulose inhibits the DNA-damaging ability of bile acids. Nutr Cancer 13(1&2):51-57.

Chen RM, Wu JJ, Lee SC, Huang AH, Wu HM (1999). Increase of intestinal *Bifidobacterium* and suppression of coliform bacteria with short-term yogurt ingestion. J Dairy Sci 82(11):2308-2314.

Chmielewska A, Ruszczynski M, Szajewska H (2008). *Lactobacillus reuteri* strain ATCC 55730 for the treatment of acute infectious diarrhoea in children: a meta-analysis of randomized controlled trials. Pediatr Wspolczesna 10(1):32-36.

Cirillo AI, Boccia E, Cirillo AR, Scotto G, Maiella D, De Crescenzo G et al. (2005). Effectiveness of *L. reuteri* in patients with atopic dermatitis and cow milk intolerance: preliminary study. Presented at: *Congresso Società di Allergologia ed Immunologia Clinica Italian Society for Clinical Allergy and Immunology (SIAIC) Congress*, May 4-7, 2005, Rome, Italy. Italian Society for Clinical Allergy and Immunology [abstract P-08]. Abstract available at: http://www.biogaia.com/health-care-professionals/clinical-evidence/other-clinical-research/allergy/atopic-dermatitis-and-co.

Cleusix V, Lacroix C, Vollenweider S, Duboux M, Le Blay G (2007). Inhibitory activity spectrum of reuterin produced by *Lactobacillus reuteri* against intestinal bacteria. BMC Microbiol 7:101. doi:10.1186/1471-2180-7-101.

Connolly E, Abrahamsson T, Bjorksten B (2005). Safety of D(-)-lactic acid producing bacteria in the human infant. J Pediatr Gastroenterol Nutr 41:489-492

Cooper DA, Berry DA, Spendel VA, King D, Kiorpes AL, Peters JC (1997). Olestra dose response on fat-soluble and water-soluble nutrients in the pig. J Nutr 127(8S):1573S-1588S.

Dambekodi PC, Gilliland SE (1998). Incorporation of cholesterol into the cellular membrane of Bifidobacterium longum. J Dairy Sci 81(7):1818-1824.

Danielson AD, Peo ER Jr, Shahani KM, Lewis AJ, Whalen PJ, Amer MA (1989). Anticholesteremic property of Lactobacillus acidophilus yogurt fed to mature boars. J Anim Sci 67(4):966-974.

De Boever P, Wouters R, Verschaeve L, Berckmans P, Schoeters G, Verstraete W (2000). Protective effect of the bile salt hydrolase-active Lactobacillus reuteri against bile salt cytotoxicity. Appl Microbiol Biotechnol 53(6):709-714.

De Rodas BZ, Gilliland SE, Maxwell CV (1996). Hypocholesterolemic action of Lactobacillus acidophilus ATCC 43121 and calcium in swine with hypercholesterolemia induced by diet. J Dairy Sci 79(12):2121-2128.

De Smet I, De Boever P, Vestraete W (1998). Cholesterol lowering in pigs through enhanced bacterial bile salt hydrolase activity. Br J Nutr 79(2):185-194.

du Toit M, Franz CMAP, Dicks LMT, Schillinger U, Haberer P, Warlies B et al. (1998). Characterisation and selection of probiotic lactobacilli for a preliminary minipig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content. Int J Food Microbiol 40(1&2):93-104.

Edwards ST, Sandine WE (1981). Public health significance of amines cheese. J Dairy Sci 64(12):2431-2438.

EFSA (2007). Opinion of the Scientific Committee on a request from EFSA on the introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. (Question no EFSA-Q-2005-293, adopted on 19 November 2007 by European Food Safety Authority). EFSA J 587:1-16. Available at: http://www.efsa.europa.eu/en/scdocs/scdoc/587.htm.

EFSA (2008). Technical guidance prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. (Question no EFSA-Q-2008-004, adopted on 18 June 2008 by European Food Safety Authority). EFSA J 732:1-15. Available at: http://www.efsa.europa.eu/en/scdocs/scdoc/732.htm.

Egervärn M, Danielsen M, Roos S, Lindmark H, Lindgren S (2007a). Antibiotic susceptibility profiles of *Lactobacillus reuteri* and *Lactobacillus* fermentum. J Food Prot 70(2):412-418.

Egervärn M, Lindmark H, Roos S, Huys G, Lindgren S (2007b). Effects of inoculum size and incubation time on broth microdilution susceptibility testing of lactic acid bacteria. Antimicrob Agents Chemother 51(1):394-396.

Elliot WH (1985). Metabolism of bile acids in liver and extrahepatic tissues (chapter 11). In: Danielsson H, Sjöval J, editors. *Sterols and Bile Acids.* (New Comprehensive Biochemistry, vol 12). New York (NY): Elsevier Science Publishers, pp. 303-329 (p. 312).

Eom T-H, Oh E-Y, Kim Y-H, Lee H-S, Jang PS, Kim D-U et al. (2005). The therapeutic effect of *Lactobacillus reuteri* in acute diarrhea in infants and toddlers. Korean J Pediatr 48(9):986-990.

Ewaschuk JB, Naylor JM, Zello GA (2005). D-lactate in human and ruminant metabolism. J Nutr 135:1619-1625

Eyssen HJ, De Pauw G, Van Eldere J (1999). Formation of hyodeoxycholic acid from muricholic acid and hyocholic acid by an unidentified gram-positive rod termed HDCA-1 isolated from rat intestinal microflora. Appl Environ Microbiol 65(7):3158-363.

FAO/WHO (2002). *Guidelines for the Evaluation of Probiotics in Food.* Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation in Food, Apr. 30-May, 1, 2002, London, Ont. Geneva, Switz.: Food and Agriculture Organization of the United Nations (FAO) / World Health Organization (WHO). Available at: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf.

Felis GE, Dellaglio F (2007). Taxonomy of lactobacilli and bifidobacteria. Curr Issues Intest Microbiol 8(2):44-61.

Ferreira IM, Pinho O (2006). Biogenic amines in Portuguese traditional foods and wines. J Food Prot 69(9):2293-2303.

Francavilla R, Lionetti E, Castellaneta SP, Magistà AM, Maurogiovanni G, Bucci N et al. (2008). Inhibition of Helicobacter pylori infection in humans by Lactobacillus reuteri ATCC 55730 and effect on eradication therapy: a pilot study. Helicobacter 13(2):127-134.

Fujiwara S, Seto Y, Kimura A, Hashiba H (2001). Intestinal transit of an orally administered streptomycin-rifampicin-resistant variant of *Bifidobacterium longum* SB52928: its long-term survival and effect on the intestinal microflora and metabolism. J Appl Microbiol 90(1):43-52.

Garai G, Duenas MT, Irastorza A, Martin-Alvarez PJ, Moreno-Arribas MV (2006). Biogenic amines in natural ciders. J Food Prot 69(12):3006-3012.

Gasser F (1994). Safety of lactic acid bacteria and their occurrence in human clinical infections. Bull Inst Pasteur 92(1):45-67.

Gilliland SE, Nelson CR, Maxwell C (1985). Assimilation of cholesterol by *Lactobacillus acidophilus.* Appl Environ Microbiol 49(2):377-381.

Govers MJ, Termont DS, Van Aken GA, Van der Meer R (1994). Characterization of the adsorption of conjugated and unconjugated bile acids to insoluble, amorphous calcium phosphate. J Lipid Res 35(5):741-748.

Grundy SM, Ahrens EH Jr, Miettinen TA (1965). Quantitative isolation and gas–liquid chromatographic analysis of total fecal bile acids. J Lipid Res 6(3):397-410.

Gu JJ, Hofmann AF, Ton-Nu HT, Schteingart CD, Mysels KJ (1992). Solubility of calcium salts of unconjugated and conjugated natural bile acids. J Lipid Res 33(5):635-646.

Gueimonde M, Ouwehand AC, Salminen S (2004). Safety of pr7obiotics. Scand J Nutr 48(1):42-48.

Guerrero M, Dohnalek M, Newton P, Kuznetsova O, Ruiz-Palacios G, Murphy T et al. (1996). Effect of probiotic-containing beverages on incidence of diarrhoea. Presented at: *1st World Congress of Pediatric Infectious Diseases*, Dec., 1996, Acapulco, Mexico. World Society of Pediatric Infectious Disease, [abstract 610:45-2]. Available at: http://www.biogaia.com/health-care-professionals/clinical-evidence/other-clinical-research/diarrhoea-reduced-risk/effect-pr ; http://www.reladrops.fi/pdf/BioGaiastudies.pdf.

Guyonnet D, Chassany O, Ducrotte P, Picard C, Mouret M, Mercier CH et al. (2007). Effect of a fermented milk containing *Bifidobacterium animalis* DN-173 010 on the health-related quality of life and symptoms in irritable bowel syndrome in adults in primary care: a multicentre, randomized, double-blind, controlled trial. Aliment Pharmacol Ther 26(3):475-486.

Hatakka K, Holma R, El-Nezami H, Suomalainen T, Kuisma M, Saxelin M et al. (2008). The influence of *Lactobacillus rhamnosus* LC705 together with *Propionibacterium freudenreichii* ssp. shermanii JS on potentially carcinogenic bacterial activity in human colon. Int J Food Microbiol 128(2):406-410.

Health Canada (2005). *Probiotics Discussion Paper*. Report submitted by Winnipeg (MB): Nutritech Consulting to Ottawa (ON): Health Canada, Natural Health Products Directorate (NHPD). Available at: http://www.hc-sc.gc.ca/dhp-mps/alt_formats/hpfb-dgpsa/pdf/pubs/probio_discuss-travail-eng.pdf.

Health Canada (2006). *Evidence for Safety and Efficacy of Finished Natural Health Products, version 2.0*. Ottawa (ON): Health Canada, Natural Health Products Directorate (NHPD). Available at: http://www.hc-sc.gc.ca/dhp-mps/alt_formats/hpfb-dgpsa/pdf/prodnatur/efe-paie-2006-eng.pdf.

Hofmann AF (1999). The continuing importance of bile acids in liver and intestinal disease. Arch Intern Med 159(22):2647-2658.

Hofmann AF (2009a). The enterohepatic circulation of bile acids in mammals: form and functions. Front Biosci 14:2584-2598.

Hofmann AF (2009b). Chronic diarrhea caused by idiopathic bile acid malabsorption: an explanation at last. Expert Rev Gastroenterol Hepatol 3(5):461-464.

Huang Y, Wang J, Cheng Y, Zheng Y (2010). The hypocholesterolaemic effects of Lactobacillus acidophilus American type culture collection 4356 in rats are mediated by the down-regulation of Niemann-Pick C1-like 1. Br J Nutr 104(6):807-812.

Hudson M, Pocknee R, Mowat NAG (1990). D-Lactic acidosis in short bowel syndrome - an examination of possible mechanisms. Q J Med 74(274):157-163.

Imase K, Tanaka A, Tokunaga K, Sugano H, Ishida H, Takahashi S (2007). *Lactobacillus reuteri* tablets suppress *Helicobacter pylori* infection–a double-blind randomised placebo-controlled cross-over clinical study. Kansenshogaku Zasshi 81(4):387-393.

Indrio F, Riezzo G, Raimondi F, Bisceglia M, Cavallo L, Francavilla R (2008). The effects of probiotics on feeding tolerance, bowel habits, and gastrointestinal motility in preterm newborns. J Pediatr 152(6):801-806.

Indrio F, Riezzo G, Raimondi F, Bisceglia M, Cavallo L, Francavilla R (2009). Effects of probiotic and prebiotic on gastrointestinal motility in newborns. J Physiol Pharmacol 60(Suppl. 6):27-31.

ISO 10932 (2010). *Draft International Standards ISO/DIS 10932 IDF 223: Milk and milk products. Determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB).* Geneva, Switz.: International Organization for Standardization (ISO).

Jacobsen CN, Rosenfeldt Nielsen V, Hayford AE, Moller PL, Michaelsen KF, Paerregaard A et al. (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. Appl Environ Microbiol 65(11):4949-4956.

Jakobsson T, Abrahamsson T, Bjorksten B, Fredrikson M, Bottcher M (2005). The effect of oral supplementation of Lactobacillus reuteri on the immunological composition of breast milk. J Pediatr Gastroenterol Nutr 40:624 [abstract OP4-05].

JECFA (1979). Polyethylene glycols. In: *Toxicological Evaluation of Certain Food Additives.* 23rd Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Apr. 2-11, 1979, Geneva, Switz. (WHO Food Additives Series, no 14). Geneva, Switz.: World Health Organization (WHO), pp. 76-83. Available at: http://www.inchem.org/documents/jecfa/jecmono/v14je19.htm.

JECFA (1992). 31.5 Thickening agents: Alginic acid and its ammonium, calcium, potassium, and sodium salts. In: *Evaluation of Certain Food Additives and Naturally Occurring Toxicants.* Thirty-ninth Report of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Feb. 3-12, 1992, Rome, 1992. (WHO Technical Report Series, no 828). Geneva, Switz.: World Health Organization (WHO), p. 22. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_828.pdf.

Jeun J, Kim S, Cho SY, Jun HJ, Park HJ, Seo, JG et al. (2010). Hypocholesterolemic effects of *Lactobacillus plantarum* KCTC3928 by increased bile acid excretion in C57BL/6 mice. Nutrition 26(3):321-330.

Johansson M-L, Molin G, Jeppsson B, Nobaek S, Ahrné S, Bengmark S (1993). Administration of different *Lactobacillus* strains in fermented oatmeal soup: in vivo colonization of human intestinal mucosa and effect on the indigenous flora. Appl Environ Microbiol 59(1):15-20.

Johnson BJ, Lee JY, Pickert A, Urbatsch IL (2010). Bile acids stimulate ATP hydrolysis in the purified cholesterol transporter ABCG5/G8. Biochemistry 49(16):3403-3411.

Jones BV, Begley M, Hill C, Gahan CG, Marchesi JR (2008). Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. Proc Natl Acad Sci U S A 105(36):13580-13585.

Jones ML, Martoni CJ, Prakash S (2011). Cholesterol lowering efficacy of microencapsulated BSH-active *Lactobacillus reuteri* cardioviva NCIMB 3042 yogurt formation in humans. Am J Clin Nutr [submitted for publication – under peer review].

Karton M, Rettmer RL, Lipkin EW (1987). Effect of parenteral nutrition and enteral feeding on D-lactic acidosis in a patient with short bowel. JPEN J Parenter Enteral Nutr 11(6):586-589.

Karvonen A, Casas I, Vesikari T (2001). Safety and possible antidiarrhoeal effect of the probiotic *Lactobacillus reuteri* after oral administration to neonates. Clin Nutr 20(Suppl. 3):63 [abstract 216].

Kaushik JK, Kumar A, Duary RK, Mohanty AK, Grover S, Batish VK (2009). Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. PLoS One 4(12):e8099 [doi: 10.1371/journal.pone.0008099].

Kawai Y, Ishii Y, Arakawa K, Uemura K, Saitoh B, Nishimura J et al. (2004). Structural and functional differences in two cyclic bacteriocins with the same sequences produced by lactobacilli. Appl Environ Microbiol 70(5):2906-2911.

Klaver FA, van der Meer R (1993). The assumed assimilation of cholesterol by Lactobacilli and Bifidobacterium bifidum is due to their bile salt-deconjugating activity. Appl Environ Microbiol 59(4):1120-1124.

Kozoni V, Tsioulias G, Shiff S, Rigas B (2000). The effect of lithocholic acid on proliferation and apoptosis during the early stages of colon carcinogenesis: differential effect on apoptosis in the presence of a colon carcinogen. Carcinogenesis 21(5):999-1005.

Krag E Phillips SF (1974). Active and passive bile acid absorption in man. perfusion studies of the ileum and jejunum. J Clin Invest 53(6):1686-1694.

Krasse P, Carlsson B, Dahl C, Paulsson A, Nilsson A, Sinkiewicz G (2006). Decreased gum bleeding and reduced gingivitis by the probiotic Lactobacillus reuteri. Swed Dent J 30(2):55-60.

Kumar R, Grover S, Batish VK (2011). Hypocholesterolaemic effect of dietary inclusion of two putative probiotic bile salt hydrolase-producing Lactobacillus plantarum strains in Sprague-Dawley rats. Br J Nutr 105(4):561-573.

Landete JM, Pardo I, Ferrer S (2007). Tyramine and phenylethylamine production among lactic acid bacteria isolated from wine. Int J Food Microbiol 115(3):364-368.

Lee DY, Seo Y-S, Rayamajhi N, Kang ML, Lee SI, Yoo HS (2009a). Isolation, characterization, and evaluation of wild isolates of *Lactobacillus reuteri* from pig feces. J Microbiol 47(6):663-672.

Lee DK, Jang S, Baek HY, Kim MJ, Lee KS, Shin HS et al. (2009b). Lactic acid bacteria affect serum cholesterol levels, harmful fecal enzyme activity, and fecal water content. Lipds Health Dis 8:21. doi: 10.1186/1476-511X-8-21.

Lehane L, Olley J (2000). Histamine fish poisoning revisited. Int J Food Microbiol 58(1&2):1-37.

Lepercq P, Relano P, Cayuela C, Juste C (2004). Bifidobacterium animalis strain DN-173 010 hydrolyses bile salts in the gastrointestinal tract of pigs. Scand J Gastroenterol 39(12):1266-1271.

Lepercq P, Hermier D, David O, Michelin R, Gibard C, Beguet F et al. (2005). Increasing ursodeoxycholic acid in the enterohepatic circulation of pigs through the administration of living bacteria. Br J Nutr 93(4):457-469.

Lionetti E, Miniello VL, Castellaneta SP, Magistá AM, De Canio A, Maurogiovanni G et al. (2006). *Lactobacillus reuteri* therapy to reduce side-effects during anti-*Helicobacter pylori* treatment in children: a randomized placebo controlled trial. Aliment Pharmacol Ther 24(10):1461-1468.

Lorea Baroja M, Kirjavainen PV, Hekmat S, Reid G (2007). Anti-inflammatory effects of probiotic yogurt in inflammatory bowel disease patients. Clin Exp Immunol 149(3):470-479.

Madsen D, Beaver M, Chang L, Bruckner-Kardoss E, Wostmann B (1976). Analysis of bile acids in conventional and germfree rats. J Lipid Res 17(2):107-711.

Marshall VM, Taylor E (1995). Ability of neonatal human Lactobacillus isolates to remove cholesterol from liquid media. Int J Food Sci Technol 30(5):571-577.

Marteau P (2001). Safety Aspects of probiotic products. Scand J Nutr 45:22-24.

Marteau P, Cuillerier E, Meance S, Gerhardt MF, Myara A, Bouvier M et al. (2002). *Bifidobacterium animalis* strain DN-173 010 shortens the colonic transit time in healthy women: a double-blind, randomized, controlled study. Aliment Pharmacol Ther 16(3):587-593.

Martinez RCR, Franceschini SA, Patta MC, Quintana SM, Candido RC, Ferreira JC et al. (2009). Improved treatment of vulvovaginal candidiasis with fluconazole plus probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14. Lett Appl Microbiol 48(3):269-274.

Micropharma (2009) [unpublished]. *In Vivo Evaluation of Microencapsulated L. reuteri Cardiovivo NCIMB 30242: Safety and Efficacy*. CONFIDENTIAL. Montreal (PQ): Micropharma Limited.

Miki M, Ishikawa T, Okayama H (2005). An outbreak of histamine poisoning after ingestion of the ground saury paste in eight patients taking isoniazid in tuberculous ward. Intern Med 44(11):1133-1136.

Mohan JC, Arora R, Khalilullah M (1990). Preliminary observations on effect of *Lactobacillus sporogenes* on serum lipid levels in hypercholesterolemic patients. Indian J Med Res 92:431-432.

Morelli L, Zonenenschain D, Del Piano M, Cognein P (2004). Utilization of the intestinal tract as a delivery system for urogenital probiotics. J Clin Gastroenterol 38(Suppl. 2):S107-S110.

Nguyen TD, Kang JH, Lee MS (2007). Characterization of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. Int J Food Microbiol 113(3):358-361.

Nikawa H, Makihira S, Fukushima H, Nishimura H, Ozaki Y, Ishida K et al. (2004). Lactobacillus reuteri in fermented bovine milk decreases the oral carriage of mutans streptococci. Int J Food Microbiol 95(2):219-223.

Niv E, Naftali T, Hallak R, Vaisman N (2005). The efficacy of *Lactobacillus reuteri* ATCC 55730 in the treatment of patients with irritable bowel syndrome-a double blind, placebo-controlled, randomized study. Clin Nutr 24(6):925-931.

Ohnuma S, Higa M, Hamanaka S, Matsushima K, Yamamuro W (2001). An outbreak of allergy-like food poisoning. Intern Med 40(8):833-835.

Ojetti V, Gigante G, Gabrielli M, Ainora ME, Mannocci A, Lauritano EC et al. (2010). The effect of oral supplementation with *Lactobacillus reuteri* or tilactase in lactose intolerant patients: randomized trial. Eur Rev Med Pharmacol Sci 14(3):163-170 [Preprint].

Ooi LG, Ahmad R, Yuen KH, Liong MT (2010). Lactobacillus acidophilus CHO-220 and inulin reduced plasma total cholesterol and low-density lipoprotein cholesterol via alteration of lipid transporters. J Dairy Sci 93(11):5048-5058.

Ouwehand AC, Lagström H, Suomalainen T, Salminen S (2002). Effect of probiotics on constipation, fecal azoreductase activity and fecal mucin content in the elderly. Ann Nutr Metab 46(3&4):159-162.

Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M et al. (2005). The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. Nucleic Acids Res 33(17):5691-5702.

Pan DD, Zeng XQ, Yan YT (2011). Characterisation of Lactobacillus fermentum SM-7 isolated from koumiss, a potential probiotic bacterium with cholesterol-lowering effects. J Sci Food Agric 91(3):512-518. doi: 10.1002/jsfa.4214.

Park YH, Kim JG, Shin YW, Kim SH, Whang KY (2007). Effect of dietary inclusion of *Lactobacillus acidophilus* ATCC 43121 on cholesterol metabolism in rats. J Microbiol Biotechnol 17(4):655-662.

Park YH, Kim JG, Shin YW, Kim HS, Kim Y-J, Chun T et al. (2008). Effects of *Lactobacillus acidophilus* 43121 and a mixture of lactobacillus casei and *Bifidobacterium longum* on the serum cholesterol level and fecal sterol excretion in hypercholesterolemia-induced pigs. Biosci Biotechnol Biochem 72(2):595-600.

Pereira DI, McCartney AL, Gibson GR (2003). An in vitro study of the probiotic potential of a bile-salt-hydrolyzing Lactobacillus fermentum strain, and determination of its cholesterol-lowering properties. Appl Environ Microbiol 69(8):4743-4752.

Pineiro M, Stanton C (2007). Probiotic bacteria: legislative framework-requirements to evidence basis. J Nutr 137(3, Suppl. 2):850S-853S.

Pochart P, Marteau P, Bouhnik Y, Goderel L, Bourlioux P, Rambaud J-H (1992). Survival of bifidobacteria ingested via fermented milk during their passage through the human small intestine: an in vivo study using intestinal perfusion. Am J Clin Nutr 55(1):78-80.

Ponz de Leon M, Loria P, Carulli N, Murphy GM, Dowling RH (1980). Intestinal solubilization, absorption, pharmacokinetics and bioavailability of chenodeoxycholic acid. Eur J Clin Invest 10(4):261-271.

Reid G, Beuerman D, Heinemann C, Bruce AW (2001). Probiotic *Lactobacillus* dose required to restore and maintain a normal vaginal flora. FEMS Immunol Med Microbiol 32(1):37-41.

Reid G, Charbonneau D, Erb J, Kochanowski B, Beuerman D, Poehner R et al. (2003a). Oral use of *Lactobacillus rhamnosus* GR-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women. FEMS Immunol Med Microbiol 35(2):131-134.

Reid G, Hammond J-A, Bruce AW (2003b). Effect of Lactobacilli oral supplement on the vaginal microflora of antibiotic treated patients: randomized, placebo-controlled study. Nutraceut Food 8(2):145-148.

Rhee YK, Han MJ, Choi EC, Kim DH (2002). Hypocholesterolemic activity of Bifidobacteria isolated from a healthy Korean. Arch Pharm Res 25(5):681-684.

Ridlon JM, Kang DJ, Hylemon PB (2006). Bile salt biotransformations by human intestinal bacteria. J Lipid Res 47(2):241-259.

Romeo MG, Betta P, Trovato L, Oliveri S (2006). The role of probiotics in the prevention of bacterial and Candida infections in Neonatal Intensive Care. prospective study with control group. Presented at: T*he 12th National SIN Congress (Italian Neonatology Society)*, May 28-31, 2006, Montecatini Terme. Available at: http://www.gadalenterprises.us/wp-content/uploads/2009/08/romeo-2006-reuteri-reduces-candida-gi-problems-in-neonates1.pdf.

Rosander A, Connolly E, Roos S (2008). Removal of antibiotic resistance plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain *L. reuteri* DSM 17938. Appl Environ Microbiol 74(19):6032-6040.

Rosenfeldt V, Michaelsen KF, Jakobsen M, Larsen CN, Moller P L, Pedersen P et al. (2002a). Effect of probiotic *Lactobacillus* strains in young children hospitalized with acute diarrhea. Pediatr Infect Dis J 21(5):411-416.

Rosenfeldt V, Michaelsen KF, Jakobsen M, Larsen CN, Moller P L, Tvede M et al. (2002b). Effect of probiotic *Lactobacillus* strains on acute diarrhea in a cohort of nonhospitalized children attending day-care centers. Pediatr Infect Dis J 21(5):417-419.

Rosenfeldt V, Pærregaard A, Nexman Larsen C, Lange Møller P, Tvede M, Sandstrøm B et al. (2003a). Faecal recovery, mucosal adhesion, gastrointestinal effects and tolerance of mixed cultures of potential probiotic lactobacilli. Microb Ecol Health Dis 15(1):2-9.

Rosenfeldt V, Benfeldt E, Nielsen SD, Michaelsen KF, Jeppesen DL, Valerius NH et al. (2003b). Effect of probiotic *Lactobacillus* strains in children with atopic dermatitis. J Allergy Clin Immunol 111(2):389-395.

Rosenfeldt V, Benfeldt E, Valerius NH, Paerregaard A, Michaelsen KF (2004). Effect of probiotics on gastrointestinal symptoms and small intestinal permeability in children with atopic dermatitis. J Pediatr 145(5):612-616.

Ruiz-Palacios G, Tuz F, Arteaga F, Guerrero ML, Dohnalek M, Hilty M (1996a). Tolerance and fecal colonization with *Lactobacillus reuteri* in children fed a beverage with a mixture of *Lactobacillus* spp.. Pediatr Res 39:184 [abstract 1090].

Ruiz-Palacios G, Guerrero M, Hilty M, Dohnalek M, Newton P, Calva J et al. (1996b). Feeding of a probiotic for the prevention of community-acquired diarrhea in young Mexican children. Pediatr Res 39(4):184 [abstract 1089].

Saggioro A, Caroli M, Pasini M, Bortoluzzi F, Girardi L, Pilone G (2005). *Helicobacter pylori* eradication with *Lactobacillus reuteri*: a double blind placebo-controlled study. Dig Liver Dis 37(Suppl. 1):S88 [abstract PO1.49].

Salminen S, von Wright A, Morelli L, Marteau P, Brassart D, de Vos WM et al. (1998). Demonstration of safety of probiotics - A review. Int J Food Microbiol 44(1&2):93-106.

Salminen MK, Tynkkynen S, Rautelin H, Poussa T, Saxelin M, Ristola M et al. (2004). The efficacy and safety of probiotic *Lactobacillus rhamnosus* GG on prolonged, noninfectious diarrhea in HIV Patients on antiretroviral therapy: a randomized, placebo-controlled, crossover study. HIV Clin Trials 5(4):183-191.

Savino F, Palumeri E, Pelle E, Castagno E, Oggero R, Miniero R (2007). *Lactobacillus reuteri* (American type culture collection strain 55730) versus simethicone in the treatment of infantile colic: a prospective randomised study. Pediatrics 119(1):e124-e130.

Saxelin M, Chuang NH, Chassy B, Rautelin H, Makela PH, Salminen S et al. (1996a). Lactobacilli and bacteremia in southern Finland, 1989-1992. Clin Infect Dis 22(3):564-566.

Saxelin M, Rautelin H, Salminen S, Mäkelä PH (1996b). Safety of commercial products with viable *Lactobacillus* strains. Infect Dis Clin Pract 5(5):331-335.

Schiff ER, Small NC, Dietschy JM (1972). Characterization of the kinetics of the passive and active transport mechanisms for bile acid absorption in the small intestine and colon of the rat. J Clin Invest 51(6):1351-1362.

Scully TB, Kraft SC, Carr WC, Harig JM (1989). D-lactate-associated encephalopathy after massive small-bowel resection. J Clin Gastroenterol 11(4):448-451.

Setchell KD, Lawson AM, Tanida N, Sjövall J (1983). General methods for the analysis of metabolic profiles of bile acids and related compounds in feces. J Lipid Res 24(8):1085-1100.

Shane AL, Cabana MD, Vidry S, Merenstein D, Hummelen R, Ellis CL et al. (2010). Guide to designing, conducting, publishing and communicating results of clinical studies involving probiotic applications in human participants. Gut Microbes 1(4):243-253.

Shornikova A-V, Casas IA, Isolauri E, Mykkänen H, Vesikari T (1997a). *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children. J Pediatr Gastroenterol Nutr 24(4):399-404.

Shornikova A-V, Casas IA, Mykkänen H, Salo E, Vesikari T (1997b). Bacteriotherapy with *Lactobacillus reuteri* in rotavirus gastroenteritis. Pediatr Infect Dis J 16(12):1103-1107.

Snydman DR (2008). The safety of probiotics. Clin Infect Dis 46(Suppl. 2):S104-S111.

Sriramulu DD, Liang M, Hernandez-Romero D, Raux-Deery E, Lünsdorf H, Parsons JB et al. (2008). *Lactobacillus reuteri* DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1,2-propanediol by disproportionation. J Bacteriol 190(13):4559-4567.

Stalker MJ, Towner RA, Kocal TE, Quinn BA (1994), Cameron RG, Hayes MA. Influences of dietary deoxycholic acid on progression of hepatocellular neoplasms and expression of glutathione S-transferases in rats. Toxicol Pathol 22(6):579-588.

St-Onge MP, Farnworth ER, Jones PJ (2000). Consumption of fermented and nonfermented dairy products: effects on cholesterol concentrations and metabolism. Am J Clin Nutr 71(3):674-681.

Sugawara G, Nagino M, Nishio H, Ebata T, Takagi K, Asahara T et al. (2006). Perioperative synbiotic treatment to prevent postoperative infectious complications in biliary cancer surgery: a randomized controlled trial. Ann Surg 244(5):706-714.

Suzzi G, Gardini F (2003). Biogenic amines in dry fermented sausages: a review. Int J Food Microbiol 88(1):41-54.

Tahri K, Grill JP, Schneider F (1996). Bifidobacteria strain behavior toward cholesterol: coprecipitation with bile salts and assimilation. Curr Microbiol 33(3):187-193.

Tahri K, Grill JP, Schneider F (1997). Involvement of trihydroxyconjugated bile salts in cholesterol assimilation by bifidobacteria. Curr Microbiol 34(2):79-84.

Tamura M, Hori S, Nakagawa H (2009). Lactobacillus collinoides JCM1123(T): effects on mouse plasma cholesterol and isoflavonoids in the caecum. Antonie Van Leeuwenhoek 96(4):621-626.

Taranto MP, Medici M, Perdigon G, Ruiz Holgado AP, Valdez GF (1998). Evidence for hypocholesterolemic effect of *Lactobacillus reuteri* in hypercholesterolemic mice. J Dairy Sci 81(9):2336-2340.

Taranto MP, Medici M, Perdigon G, Ruiz Holgado AP, Valdez GF (2000). Effect of *Lactobacillus reuteri* on the prevention of hypercholesterolemia in mice. J Dairy Sci 83(3):401-403.

Toba T, Samant SK, Yoshioka E, Itih T (1991). Reutericin 6, a new bacteriocin produced by *Lactobacillus reuteri* LA 6. Lett Appl Microbiol 13:281-286.

Tubelius P, Stan V, Zachrisson A (2005). Increasing work-place healthiness with the probiotic *Lactobacillus reuteri*: a randomised, double-blind placebo-controlled study. Environ Health 4:25 [doi:10.1186/1476-069X-4-25].

Twetman S, Derawi B, Keller M, Ekstrand K, Yucel-Lindberg T, Stecksén-Blicks C (2009). Short-term effect of chewing gums containing probiotic Lactobacillus reuteri on the levels of inflammatory mediators in gingival crevicular fluid. Acta Odontol Scand 67(1):19-24.

U.S. FDA (1997). Substances generally recognized as safe; Proposed rule (21 CFR Parts 170, 184, 186, and 570) [Docket No. 97N-0103]. Fed Regist (US) 62(74):18937-18964.

U.S. FDA (2001). *Partial List of Microorganisms and Microbial-Derived Ingredients That are Used in Foods*. Rockville (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN). Available at: http://www.fda.gov/Food/FoodIngredientsPackaging/ucm078956.htm [Page Last Updated: 06/22/2009].

U.S. FDA (2008). *[GRAS Notice – Substance: Lactobacillus reuteri strain DSM 17938 (GRN 254)]*. Submitted to: Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Premarket Approval, Division of Biotechnology and GRAS Notice Review (HFS-255) by Stockholm, Sweden: BioGaia AB. Available at: http://www.accessdata.fda.gov/scripts/fcn/gras_notices/808166A.PDF.

U.S. FDA (2009). Milk and cream products and yogurt products; proposal to revoke the standards for lowfat yogurt and nonfat yogurt and to amend the standard for yogurt [21 CFR Part 131; Docket No. FDA–2000–P–0126] (formerly Docket No. 2000P–0685)]. Fed Regist (US) 74(10):2443-2460.

U.S. FDA (2011a). *U.S. Code of Federal Regulations (CFR). Title 21—Food and Drugs (Food and Drug Administration)*. Washington (DC): U.S. Government Printing Office (GPO). Available at: http://www.gpo.gov/fdsys/browse/collectionCfr.action?collectionCode=CFR [See Table for CFR sections].

Table of CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
101—Food labeling	101.12	Reference amounts customarily consumed per eating occasion
170—Food additives	170.3	Definitions
	170.30	Eligibility for classification as generally recognized as safe (GRAS)
172—Food additives permitted for direct addition to food for human consumption	172.820	Polyethylene glycol (mean molecular weight 200-9,500)
184—Direct food substances affirmed as generally recognized as safe	184.1193	Calcium chloride
	184.1540	Nitrogen
	184.1724	Sodium alginate
	184.1763	Sodium hydroxide

U.S. FDA (2011b). *Agency Response Letter GRAS Notice No. GRN 000336 [ε-Polylysine ((polylysine))]*. Submitted by Gorinchem, The Netherlands: Purac Biochem b.v. to College Park (MD): U.S. Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/ucm271325.htm.

USDA (2000). *1994-1996, 1998 Continuing Survey of Food Intakes by Individuals (CSFII) and Diet and Health Knowledge Survey (DHKS)* [On CD-ROM, PB2000-500027]. Riverdale (MD): U.S. Department of Agriculture (USDA).

USDA (2009). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2005-2006*. Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release.

Usman; Hosono A (2000). Effect of administration of Lactobacillus gasseri on serum lipids and fecal steroids in hypercholesterolemic rats. J Dairy Sci 83(8):1705-1711.

Usman; Hosono A (2001). Hypocholesterolemic effect of *Lactobacillus gasseri* SBT0270 in rats fed a cholesterol-enriched diet. J Dairy Res 68(4):617-624.

Valeur N, Engel P, Carbajal N, Connolly E, Ladefoged K (2004). Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract. Appl Environ Microbiol 70(2):1176-1181.

Vankerckhoven V, Huys G, Vancanneyt M, Vael C, Klare I, Romond M-B et al. (2008). Biosafety assessment of probiotics used for human consumption: recommendations from the EU-PROSAFE project. Trends Food Sci Technol 19(2):102-114.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M et al. (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23(21):4407-4414.

Wang Y, Xu N, Xi A, Ahmed Z, Zhang B, Bai X (2009). Effects of Lactobacillus plantarum MA2 isolated from Tibet kefir on lipid metabolism and intestinal microflora of rats fed on high-cholesterol diet. Appl Microbiol Biotechnol 84(2):341-347.

Wassenaar TM, Klein G (2008). Safety aspects and implications of regulation of probiotic bacteria in food and food supplements. J Food Prot 71(8):1734-1741.

Weizman Z, Alsheikh A (2006). Safety and tolerance of a probiotic formula in early infancy comparing two probiotic agents: a pilot study. J Am Coll Nutr 25(5):415-419.

Weizman Z, Asli G, Alsheikh A (2005). Effect of a probiotic infant formula on infections in child care centers: comparison of two probiotic agents. Pediatrics 115(1):5-9.

Wolf BW, Garleb KA, Ataya DG, Casas IA (1995). Safety and tolerance of *Lactobacillus reuteri* in healthy adult male subjects. Microb Ecol Health Dis 8(2):41-50.

Wolf BW, Wheeler KB, Ataya DG, Garleb KA (1998). Safety and tolerance of *Lactobacillus reuteri* supplementation to a population infected with human immunodeficiency virus. Food Chem Toxicol 36:1085-1094.

Wutzke KD, Oetjens I (2005). 13C- and 15N-incorporation of doubly stable isotope labelled *Lactobacillus johnsonii* in humans. Eur J Clin Nutr 59(10):1167-1172.

Wutzke KD, Berg D, Haffner D (2008). The metabolic fate of doubly stable isotope labelled heat-killed *Lactobacillus johnsonii* in humans. Eur J Clin Nutr 62(2):197-202.

Xiao JZ, Kondo S, Takahashi N, Miyaji K, Oshida K, Hiramatsu A et al. (2003). Effects of milk products fermented by *Bifidobacterium longum* on blood lipids in rats and healthy adult male volunteers. J Dairy Sci 86(7):2452-2461.

APPENDIX A

Appendix A

Expert Panel Statement

Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Use of *Lactobacillus reuteri* NCIMB 30242 as a Food Ingredient in Multiple Food Categories

April 29, 2011

Micropharma Incorporated (Micropharma) proposes to market *Lactobacillus reuteri* NCIMB 30242 (*L. reuteri* NCIMB 30242), under the trade name *Lactobacillus reuteri* Cardioviva®, in the United States for use as a food ingredient in a multiple food categories (beverage and beverage bases, breakfast cereals, cheeses, dairy product analogs, fats and oils, frozen dairy desserts, grain products and pastas, milk products, processed fruits and fruit juices, soft candy, and sugar substitutes) at use levels of up to $1x10^{10}$ CFU/serving.

At the request of Micropharma, an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether uses of *Lactobacillus reuteri* NCIMB 30242 in the proposed foods described in Table A-1 (see Appendix A) are safe and suitable and would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Expert Panel consisted of Dr. Michael Pariza Ph.D. (University of Wisconsin), Dr. Stephen L. Taylor Ph.D. (University of Nebraska), and Dr. Gary M. Williams, M.D. (New York Medical College). These Panel members have been determined to be qualified by relevant experience and scientific training to evaluate the safety of *L. reuteri* NCIMB 30242 under the aforementioned proposed food uses. *Curricula vitae* for each Panel member are included in Appendix B.

The Expert Panel, independently and collectively, critically evaluated a dossier which included a summary of the scientific information on *L. reuteri* NCIMB 30242 prepared from a comprehensive search of the scientific literature and also included details pertaining to the method of manufacture and product specifications, supporting analytical data, intended use conditions of *L. reuteri* NCIMB 30242 in food, estimated exposure under the proposed uses, and a comprehensive assessment of the available scientific literature pertaining to the safety of the material. In addition, the Expert Panel evaluated other information deemed appropriate or necessary.

Following its independent, critical evaluation of such data and information, the Expert Panel convened on Friday, April 29, 2011 and unanimously concluded that the intended uses described herein for *L. reuteri* NCIMB 30242, meeting appropriate food-grade specifications as

described in the supporting dossier [**Documentation Supporting the Generally Recognized as Safe (GRAS) Use of *Lactobacillus reuteri* NCIMB 30242 as Food Ingredient for Use in a Multiple Food Categories**] and manufactured according to current Good Manufacturing Practice (cGMP), are GRAS based on scientific procedures. A summary of the basis for the Expert Panel's conclusion is provided below.

The ingredient that is the subject of this GRAS evaluation is a strain of *Lactobacillus reuteri* (NCIMB 30242) that has been selected for its high bile salt hydrolase (BSH) activity and ability to benefit cardiovascular health when consumed in adequate quantities in the diet. The organism has been characterized phenotypically using API 50 carbohydrate fermentation profile strip testing, and APIzym enzymatic analyses. The fermentation and enzymatic profile generated by *L. reuteri* NCIMB 30242 is consistent with that expected by *L. reuteri* and related species, and these methods are suitable for early stage identification of the organism. The Panel understands that McGill University & Genome Innovation Center has sequenced the genome of the organism using shotgun sequencing methods and common bioinformatic analysis techniques. Using the GenBank database and sequence comparison alignment software (BLAST), sequence data for the entire *L. reuteri* NCIMB 16S ribosomal RNA (16S rRNA) gene was compared to available 16S gene sequences within the databank; a concise alignment (≥99%) of the *L. reuteri* NCIMB 30242 gene with multiple *L. reuteri* strains was shown, and identification of the microorganism at the species level as *Lactobacillus reuteri* was concluded. Characterization of *L. reuteri* NCIMB 30242 at the strain level was conducted by third party experts (BCCM/LMG) using Amplified Fragment Length Polymorphism (AFLP) analysis. The Panel understands that this method has been validated for use with *L. reuteri* by BCCM/LMG, and considers AFLP to be a suitable genotypic method for strain characterization of many lactobacillus species. Repeated analysis of multiple samples of *L. reuteri* NCIMB 30242 generated AFLP banding profiles that were greater than 80% similar. Dendrogram analyses of the *L. reuteri* NCIMB 30242 AFLP profile with several non-related strains of *L. reuteri* produced a similarity of below 60% confirming the usefulness of the assay to characterize the organism at the strain level. Due to the availability of a large database of AFLP profiles of various *Lactobacillus* strains at BCCM/LMG, *in silico* analyses were sufficiently robust to also classify *L. reuteri* NCIMB 30242 within the species of *L. reuteri*. The Panel considered *L. reuteri* NCIMB 30242 to be adequately characterized at the strain level and would support use in quality control during production, and for post-market surveillance monitoring if required. The Panel noted that identification of *L. reuteri* NCIMB 30242 at the species level did not use gold standard genetic analyses (DNA-DNA reassociation). In addition, although 16S rRNA gene sequence analysis is widely regarded as one of the best tools for the taxonomic positioning of lactic acid bacteria, the technique has limited resolution for the discrimination of very closely related species lactic acid bacteria (*e.g.*, *L. reuteri* and *L. fermentum*). Nevertheless, the combination of 16S RNA analyses of the entire genome and AFLP comparisons against a large database of *Lactobacillus* species were sufficient to confidently conclude the identity of the organism as a member of *L. reuteri*.

The Panel noted that *L. reuteri* NCIMB 30242 is currently deposited in an international culture bank (NCIMB) under the strain identification number NCIMB 30242. This culture deposit is currently used as a master culture for preparation of all commercial Micropharma products. The Panel reviewed information provided by Micropharma describing the chemistry and manufacturing of *L. reuteri* NCIMB 30242. Fermentation of the microbe is conducted at Chr Hansen (Denmark), a company that has a long-history of providing quality microorganism preparations for use by the food industry in the United States and world-wide. The Panel reviewed documentation supporting that *L. reuteri* NCIMB 30242 is fermented under cGMP using permitted and suitable fermentation processing-aids. The Panel understands that quality control methods are implemented throughout various stages of fermentation to ensure production of a pure culture absent contaminating pathogens. Consistency of the fermentation method was supported by batch analyses from three non-consecutive lots in compliance with the product specifications. To improve stability/viability of the organism, the lyophilized microorganism may be reformulated with various food grade encapsulation ingredients. Currently sodium alginate, polyethylene glycol 1500, and Epsilon-polylysine are used. All of these materials are currently permitted for use in food in the United States. Reformulation of the ingredient is conducted at a second contract research facility (BRACE GmbH, Germany). Suitable food grade specifications also have been developed for the encapsulated material ensuring the final food grade product is of high purity and free of contaminating microbes and heavy metals. Batch analyses of three non-consecutive lots of the ingredient in compliance with product specifications were provided to the Panel.

The Expert Panel understands that *L. reuteri* NCIMB 30242 is proposed for use as a food ingredient in multiple food categories as described in Table A-2 (Appendix A) at use levels of up to up to $1x10^{10}$ CFU/serving. Estimates for the intake of Micropharma's *L. reuteri* NCIMB 30242 ingredient were based on the proposed food-uses and use-levels in conjunction with food consumption data included in the 2003-2004 and 2005-2006 NHANES (CDC, 2006, 2009; USDA, 2009), which provides the most appropriate data for evaluating food use and food consumption patterns in the U.S. Under the conditions of intended use and on an all-user basis, the mean intake of *L. reuteri* NCIMB 30242 by the total U.S. population from all proposed food-uses was estimated to be $1.8x10^{10}$ CFU/person/day. The heavy consumer (90^{th} percentile) all-user intake of *L. reuteri* NCIMB 30242 by the total U.S. population from all proposed food-uses was estimated to be $3.5x10^{10}$ CFU/person/day. On an individual population basis, the greatest mean all-user intake of *L. reuteri* NCIMB 30242 on an absolute basis was determined to occur in children and male teenagers at $2.3x10^{10}$ CFU/person/day. The Panel considered background exposure to existing food uses of *L. reuteri* in the U.S. market, and determined that it was inappropriate to include background estimates of different strains since these are expected to have strain specific phenotypes that may not be relevant to dietary exposure to Micropharma's strain. The Panel also considered exposure to the encapsulation agents under the proposed uses. Exposure to these ingredients under the proposed of *L. reuteri* NCIMB 30242 as described in table A-1 would result in limited dietary exposures, and would not appreciably

change background exposures to these ingredients from their currently permitted uses in the U.S. diet; the proposed use of these materials as encapsulation agents was therefore considered GRAS.

The Panel reviewed a large body of safety data during their assessment of the safety of *L. reuteri* NCIMB 30242; this information included both strain specific information and relevant safety data generated using non-related strains of *L. reuteri*. Although the use of *L. reuteri* for its nutritional value is a recent development, the Panel noted that strains of *L. reuteri* have a long-history of safe use in the food supply; *L. reuteri* is commonly employed by the food industry for its fermentation properties, and specifically, is one of the most widely used microorganisms for the production of sourdough breads. In addition, the presence of commensal strains of *L. reuteri* is in human breast milk has been observed among lactating women sampled across a broad world-wide geographical distribution. Corresponding presence of *L. reuteri* as a natural resident of the gastrointestinal tract of infants has been demonstrated.

Information on the metabolic fate of orally administered *L. reuteri* is not available. However, given that the ingredient is a live microorganism, it is expected to transiently colonize the small intestine and colon following consumption, largely escaping digestive and absorptive processes. Viability of the microorganism throughout the gastrointestinal tract, including the small intestine, is supported by reductions in serum cholesterol levels observed in hypercholesterolemic subjects consuming the organism on a daily basis. Organisms not surviving gastrointestinal transit would be metabolized by human digestive enzymes and the nutritive components of the cell (proteins, lipids, carbohydrates) would be used as a source of nutrients. Non-nutritive components would be further metabolized by the resident microflora of the colon, and/or excreted in the feces.

A comprehensive search of the literature did not identify toxicology studies conducted with *L. reuteri*. Strain specific toxicology studies conducted using *L. reuteri* NCIMB 30242 also have not been conducted. The Panel acknowledged that it is generally accepted that microbial-host interactions are species specific, and that validated animal toxicology models for use in hazard characterization of microbial ingredients intended for human consumption do not exist. The Panel therefore placed an emphasis on history of exposure to *L. reuteri* (background exposure in food and as a commensal organism), strain specific safety data provided by a repeat dose study in humans, and bioinformatic and *in vitro* data characterization the metabolic phenotypes of the strain. The Panel considered this information to be adequate to evaluate the safety of *L. reuteri* NCIMB 30242 under the proposed food uses.

The Panel reviewed a product specific study evaluating the consumption of *L. reuteri* NCIMB 30242 by healthy adults. The study employed a randomized placebo controlled parallel study design, and was conducted in 120 hypercholesterolemic male and female subjects. The Panel considered the study population to be representative of typical users of foods containing *L. reuteri* NCIMB 30242. The ingredient used in the trial was encapsulated as described within

Micropharma's GRAS self-affirmation dossier, and was formulated within a yogurt matrix. Daily intake of *Lactobacillus reuteri* NCIMB 30242 in the treatment subjects was $5x10^{10}$ CFU/day. Subjects consumed the test yogurts on a daily basis over a period of 6 weeks. Measurements of cholesterol and fecal microflora and standard clinical chemistry and hematology safety endpoints were obtained at baseline and upon completion of the treatment period. No adverse events were reported, and no significant between group differences in safety parameters[1] were reported between the placebo and treatment groups. Consistent with the bile salt active phenotype of the organism, a statistically significant between group reduction in serum cholesterol levels from baseline was observed in the treatment subjects. This effect was ~10%, and was considered a beneficial nutritional response, particularly given the hypercholesterolemic status of the participants. The Panel understands that a manuscript of this study is currently under peer-review for publication in the literature.

The Panel noted that a large number of human studies have been conducted with 11 different strains of *L. reuteri*. In total 59 studies were obtained and reviewed for information relevant to the safety of *L. reuteri* consumption. These studies included trials conducted in healthy adults, infants and children, and in groups of adults, infants and children with various disease states or adverse physiological conditions requiring medical intervention (*e.g.*, atopic dermatitis, acute diarrhea, rotavirus infection). Several studies also were identified that contained detailed clinical chemistry and hematology safety evaluations. Within these studies *L. reuteri* has been administered at doses of up to $1x10^{11}$ CFU/person/day, and doses of between $1x10^{8}$ and $1.2x10^{9}$ CFU/person/day have been safely administered and well tolerated by children for durations of up to 1 year. Consumption of *L. reuteri* in these studies typically results in transient colonization of the colon, with colonization rapidly diminishing following discontinuation of probiotic administration. The Panel noted that the daily intakes of *L. reuteri* evaluated in these studies were comparable to the conservative estimates of exposure to *L. reuteri* NCIMB 30242 in the diet under the proposed food uses in the United States. To date, no adverse events or severe adverse events attributable to *L. reuteri* have been reported in the literature. Based on the long-history of safe use of *L. reuteri* in food, its ubiquitous presence as a human commensal within human milk and with the gastrointestinal tract of infants, and the absence of adverse effects attributed to the consumption various non-related strains of *L. reuteri* in human studies, the Panel determined that there was sufficient evidence to conclude that the species *L. reuteri* can be generally concluded to be non-pathogenic, and non-toxigenic. This conclusion is consistent with the opinion of the European Food Safety Authority (EFSA), who evaluated the

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[1] Serum (biochemistry) was analyzed for urea, creatinine, bilirubin, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase, glucose, calcium, phosphate, potassium, sodium, chloride, bicarbonate and lipase. Whole blood (haematology) was analyzed for haemoglobin, haematocrit, red blood cells, white blood cells and platelets. Subjects also were subject to physical and clinical examinations and were monitored by a physician throughout the experiment.

safety of *L. reuteri* for use in food and feed applications, and granted a Qualified Presumption of Safety (QPS) status to the species for its existing traditional food uses.

Lactobacillus reuteri NCIMB 30242 was specifically selected for its high bile salt hydrolase activity, a metabolic phenotype that is associated with reductions in serum cholesterol in studies evaluating repeated consumption of the strain by rodents and humans. The Panel noted that numerous probiotic safety assessments and guidance documentation prepared by various regulatory bodies have cautioned against the use of bile salt active microorganisms as food ingredients on the basis that residence of bile salt active microbes proximal to the terminal ileum may result in increased colonic concentrations of bile acids. Increased local and systemic exposure to "toxic" secondary bile acids may be undesirable since secondary bile acids are considered the etiological agents in the epidemiological association between fat intake and the incidence of colon cancer among consumers of high fat diets. The concern that consumption of bile salt active microorganisms could result in increased transport of bile acids to the colon is based exclusively on the belief that deconjugation of bile acids proximal to the terminal ileum reduces their solubility and prevents active transport of the molecules in the terminal ileum, which in-turn results in increased colonic concentrations of bile acids that are subject to secondary microbial biotransformation events. A comprehensive and critical review of the literature was therefore conducted by Micropharma to identify studies characterizing the metabolic fate and gastrointestinal physiology of bile acids in humans and animals. Additional studies evaluating the effect of BSH-active microorganism consumption in rats, pigs, and humans also were evaluated for evidence of increased fecal and systemic exposure to secondary bile acids when these organism are consumed on a repeated basis at high dietary levels. Based on a critical review of the aforementioned information, it was determined that concerns related to loss of deconjugated bile salts in the feces associated with deconjugation of bile acids proximal to the terminal ileum are not adequately supported by references to the primary literature. More importantly, claims that deconjugated bile acids are not well absorbed in the small intestine are inconsistent with the available scientific information characterizing bile acid metabolism and physiology in animals, which shows that deconjugated bile acids are well absorbed in the small intestine by both passive and active transport processes; absorption is typically equivalent to or greater than that observed with their conjugated counterparts. The repeated administration of high dietary concentrations of bile-acid hydrolase active microorganism in conjunction with a high fat diet has been evaluated by several authors using rats and pigs. Although the data reported are conflicting, the Panel noted that observations of increased fecal and systemic concentrations of bile acids were largely limited studies conducted in rats, and typically involved the use of non-validated, and non-qualitative colorimetric assays for measurement of bile acids. Studies in pigs in which qualitative methods of bile acid analysis (Gas chromatography/mass spectroscopy) were employed, did not identify increased fecal concentrations of bile acids following consumption of bile acid hydrolase microbes in conjunction with high fat diets. Based on the similarity of the pig gastrointestinal anatomy and physiology to humans, the Panel placed emphasis on findings is this animal model over the rat.

Two studies were indentified in which fecal bile acids were measured in subjects administered bile salt active microorganisms in the diet for durations of between 10 days to 3 months. The effects of *L. reuteri* NCIMB 30242 consumption on fecal bile acids were evaluated during Micropharma's placebo controlled human study, and the data were available to the Panel. In all of these studies fecal bile acid concentrations were evaluated *via* gas chromatography or a combination of gas chromatography mass spectroscopy; no evidence of increased fecal concentrations of bile acids was observed in the subjects randomized to the groups administered bile salt active microorganisms. Thus, based on studies conducted in pigs and humans (including a product specific study conducted with *L. reuteri* NCIMB 30242) administered bile acid hydrolase active microorganisms in the diet, which included sensitive and qualitative measures for analyses of fecal and serum bile acids, the Panel considered cautions within the literature pertaining to the consumption of bile salt hydrolase active microorganism to be unsubstantiated, and inconsistent with the current understanding of bile acid physiology. The Panel also noted that the safety concerns related to the expression of bile salt hydrolase by microorganisms intended for addition to food was previously evaluated by a qualified panel of Experts during a recent workshop as part of European Union PROSAFE project. The workshop consisted of 60 academic and industry scientists who convened to discuss recommendations on taxonomy, antibiotic resistance, *in vitro* assessment of virulence and *in vivo* assessment of safety of probiotics used for human consumption. The PROSAFE Panel concluded "...that bile salt deconjugation activity is irrelevant for safety assessment and is not recommended for *in vitro* safety testing of probiotics."

Consistent with the BSH active phenotype of *L. reuteri* NCIMB 30242, regular consumption of the microorganisms is associated with cholesterol reduction properties when administered to rodents and hypercholesterolemic humans. The mechanism for this effect is unclear, and several possibilities including disruption of bile acid enterohepatic circulation, and impairments in cholesterol absorption through reduced efficiency of mixed micelle formation have been suggested. Recent studies have shown that through interactions with lipids and cholesterol, de-conjugated bile acids can effectively stabilize the adenosine triphosphate binding cassette (ABC) cholesterol efflux transporter leading to a reductions in the absorption efficiency of dietary cholesterol and potentially increased excretion of circulating cholesterol into the bile, both processes leading to a net loss of cholesterol into the feces. The Panel considered the level of cholesterol reduction observed during consumption of *L. reuteri* NCIMB 30242 and other bile salt hydrolase active microorganisms to be comparable to that observed with other GRAS food ingredients that are (phytosterol/stanol preparations, oat and barley glucans) widely used by the general U.S population. Similar to phytosterol-containing foods, food products containing *L. reuteri* NCIMB 30242 will be marketed for their nutritional benefit to cardiovascular health, and are therefore unlikely to consumed on a regular basis by non-target users; sporadic use of *L. reuteri* NCIMB 30242 in these individual is not expected to affect cholesterol metabolism since the organism displays transient residence in the colon, and requires repeated consumption to exert effects on cholesterol metabolism. Safety concerns related to exposure of

sensitive population groups (children and pregnant women) to cholesterol lowering foods were not identified; in fact the Panel recognized an increasing nutritional need to safely manage hypercholesterolemia in these population groups.

The antibiotic resistance profile of a microorganism is a strain specific phenotype, and *in vitro* antibiotic resistance testing has been conducted for *L. reuteri* NCIMB 30242 against a broad range of clinically important antibiotics using validated agar diffusion methods. *L. reuteri* NCIMB 30242 observed to be susceptible to all antibiotics tested. This information is published within the peer-reviewed literature. One published study was identified by Micropharma in which the susceptibility of *L. reuteri* NCIMB 30242 to chlormaphenical deviated outside the corresponding breakpoint value of the antibiotic established for *L. reuteri* by EFSA. The apparent deviation in the MIC values relative to the breakpoint MIC value of 4 µg/mL was slight and not consistently observed. Using available gene sequence data, a bioinformatic analyses was conducted to identify potential chloramphenicol resistance determinants. Matches of the gene sequence data to homologs of chloramphenicol resistance genes were limited to a multi-drug transporter that was 23% identical to the chloramphenicol transporter FloR. It was therefore determined that the apparent chloramphenicol resistance is due to conditional difference in chloramphenicol uptake, and not a result of an acquired and potentially transmissible antibiotic resistance gene. Gene sequence analyses did not identify any transmissible elements with the gene sequence of *L. reuteri* NCIMB 30242. The Panel noted that chloramphenicol is no longer widely used in Western medical practice, and concluded that the apparent chloramphenicol resistance was not a safety concern.

Several additional metabolic phenotypes of the organism including the production of D-lactic acid, biogenic amines, and antimicrobials, were investigated, and this information is generally available. *Lactobacillus reuteri* NCIMB 30242 was shown to ferment lactose to a racemic mixture of D- and L-lactic acid in a ratio of 45:55% respectively. Although D-lactate production by the gut microflora has been reported to result in D-lactic acidosis in subjects with short bowel syndrome, the effect is limited to this pathological condition, and always in association with antibiotic use and consumption of a high carbohydrate diet. Humans can actively metabolize D-lactose, and studies conducted in infants consuming a strain of *L. reuteri* that produces quantitatively comparable levels of D-lactic acid did not result in elevations in serum D-lactate concentrations outside the normal range. Micropharma noted that safety concerns related to D-lactic acidosis resulting from the consumption of D-lactic acid producing microorganisms were comprehensively evaluated by Biogaia during their GRAS self-affirmation of *L. reuteri* DSM 17938, a microorganism that produces a similar racemic mixture of D- and L- lactic acid during fermentation of lactose. Based on generally available information, Biogaia concluded that production of D-lactic acid by *L. reuteri* DSM 17938 was not a safety concern under their proposed food uses; this position was not questioned by the United States Food and Drug Administration during review of the companies GRAS Notification, which received a letter of no

objection from the Agency. The Panel considered this view to be appropriate and applicable to the use of *L. reuteri* NCIMB 30242 in food.

The capacity of NCIMB 30242 to synthesize biogenic amines was investigated under culture conditions optimized for biogenic amine production. Microparma used colorimetric indicator assays and HPLC broth analyses for detection of biogenic amines production by the organism. No colorimetric staining was observed in colonies of *L. reuteri* NCIMB 30242 grown in biogenic amine indicator media, and the high performance liquid chromatographic (HPLC) analyses of fermentation broth for putrescine, cadaverine, histamine, and tyramine were negative at a detection level of 1 mg/L.

Strains of *L. reuteri* are known to produce various antimicrobial products including a tetrameric acid derivative, reutericin, and a broad spectrum antimicrobial reuterin that is synthesized from glycerol. Neither production of reutericin, nor reuterin, could be detected in the fermentation media of *L. reuteri* NCIMB 30242.

Finally, a bioinformatic search of the annotated gene sequence was conducted to identify any potentially undesirable phenotypic properties of the organism. Forty percent of the identified genes were classified as established sub-systems based on functional roles in metabolic pathways or class of protein. Sixteen genes classified as virulence genes were identified. Four genes were associated with iron scavenging mechanisms and 12 genes were classified in the antibiotic resistance and toxic compound sub-systems. These genes were determined to be common to lactobacilli and were not associated with mobile elements, and therefore were concluded to be of no safety concern. No genes involved in pathogen adhesion, invasion, toxin or superantigen production, or regulation of virulence were identified in the analysis.

CONCLUSION

We, the members of the Expert Panel, have independently and collectively critically evaluated the information summarized above and conclude that *Lactobacillus reuteri* NCIMB 30242, meeting appropriate food-grade specifications and manufactured in accordance with current Good Manufacturing Practice, is Generally Recognized as Safe, based on scientific procedures, for use within the proposed food categories described within Table A-1.

It is our opinion that other qualified experts would concur with these conclusions.

(b) (6)

6 July 2011

Prof. Michael W. Pariza, Ph.D.
Food Research Institute
Department of Food Science
University of Wisconsin

Date

(b) (6)

12 July 2011

Prof. Stephen L. Taylor, Ph.D.
Food Science and Technology
University of Nebraska

Date

(b) (6)

18 July 2011

Prof. Gary M Williams
Department of Pathology
New York Medical College

Date

References

CDC (2006). *Analytical and Reporting Guidelines: The National Health and Nutrition Examination Survey (NHANES)*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: http://www.cdc.gov/nchs/data/nhanes/nhanes_03_04/nhanes_analytic_guidelines_dec_2005.pdf.

CDC (2009). *National Health and Nutrition Examination Survey (NHANES): 2005-2006*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: http://www.cdc.gov/nchs/nhanes/nhanes2005-2006/nhanes05_06.htm.

U.S. FDA (2010). Part 101—Food labeling. §101.12—Reference amounts customarily consumed per eating occasion. In: *U.S. Code of Federal Regulations (CFR). Title 21—Food and Drugs (Food and Drug Administration)*. Washington (DC): U.S. Government Printing Office (GPO), pp. 46-56. Available at: http://www.access.gpo.gov/nara/cfr/cfr-table-search.html#page1.

USDA (2009). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2005-2006*. Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release.

APPENDIX A

Individual Proposed Food-Uses and Use-Levels for *Lactobacillus reuteri* NCIMB 30242™ in the U.S.

Table A-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *Lactobacillus reuteri* NCIMB 30242™ in the United States (2003-2006 NHANES Data)

Food Category	Proposed Food-Uses	Reuteri Use Level (g/serving) (CFU/serving)	Serving Size (g or mL)*	Reuteri Use-Level (%)
Beverages and Beverage Bases	Meal Replacement Beverages	$1x10^{10}$	240	0.17
Breakfast Cereals	Ready-to-Eat Breakfast Cereals	$1x10^{10}$	15 (Puffed)	2.67
			30 (Regular)	1.33
			55 (Biscuit-Type)	0.73
Cheeses	Cream Cheese[a]	$3.3x10^{9}$	30	0.43
	Natural Cheese[a]	0.13 $3.3x10^{9}$	30	0.43
	Processed Cheese and Spreads[a]	$3.3x10^{9}$	30	0.43
Dairy Product Analogs	Soy-Based Beverages	$1x10^{10}$	240	0.17
Fats and Oils	Butter	$3.3x10^{9}$	15	0.87
	Fat-Based Sauces	$3.3x10^{9}$	30	0.43
	Margarine[a] and Margarine-like Spreads	$3.3x10^{9}$	15	0.87
	Mayonnaise[a] and Mayonnaise-Type Dressings	$3.3x10^{9}$	15	0.87
	Salad Dressings[a]	$3.3x10^{9}$	30	0.43
	Vegetable Oils	$3.3x10^{9}$	15	0.87
Frozen Dairy Desserts	Frozen Novelties and Frozen Milk Desserts	$1x10^{10}$	120	0.33
	Frozen Yogurt	$1x10^{10}$	120	0.33
	Ice Cream[a]	$1x10^{10}$	120	0.33
			240 (sundaes)	0.17
Grain Products and Pastas	Cereal and Granola Bars	$1x10^{10}$	40	1.00
	Energy, Meal Replacement, and Fortified Bars	$1x10^{10}$	40	1.00
Milk Products	Fermented Milks (plain)[a]	$1x10^{10}$	240	0.17
	Flavored Milk, Milk Drinks, and Mixes	$1x10^{10}$	120 (eggnog)	0.33
			240	0.17
	Milk-Based Meal Replacement Beverages	$1x10^{10}$	240	0.17
	Sour Cream[a]	$3.3x10^{9}$	30	0.43
	Yogurt[a]	$1x10^{10}$	225	0.18
	Yogurt Drinks[b]	$1x10^{10}$	240	0.17
Processed Fruits and Fruit Juices	Fruit Juices	$1x10^{10}$	240	0.17

Table A-1 Summary of the Individual Proposed Food-Uses and Use-Levels for *Lactobacillus reuteri* NCIMB 30242™ in the United States (2003-2006 NHANES Data)

Food Category	Proposed Food-Uses	Reuteri Use Level (g/serving) (CFU/serving)	Serving Size (g or mL)*	Reuteri Use-Level (%)
Soft Candy	Chocolate Confectionary	$3.3x10^9$	40	0.33
Sugar Substitutes	Sugar Substitutes	$3.3x10^9$	4	3.25

* Serving sizes were based on Reference Amounts Customarily Consumed (RACC) per Eating Occasion in the U.S. CFR (21 CFR §101.12) (U.S. FDA, 2010).
[a] These food-uses represent non-standardized food products; however, in order to obtain a conservative intake estimate, surrogate codes for the standardized food products were chosen.
[b] No food codes were identified for yogurt drinks; therefore, surrogate codes of fruit smoothie drinks were used to represent the food codes in this category. CFU = (colony forming unit).

APPENDIX B

Appendix B

Additional Information

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Dairy Cultures

EU Allergen Information

Material No: 701359

Version: 3, November 2010

List of common allergens in accordance with US Food Allergen Labelling and Consumer Protection Act of 2004 (FALCA) and EU labelling Directive 2000/13/EC with later amendments	Present as an ingredient in the product	Ingredient species or type
Cereals containing gluten* and products thereof	NO	
Crustacean and products thereof	NO	
Eggs and products thereof	NO	
Fish and products thereof	NO	
Peanuts and products thereof	NO	
Soybeans and products thereof	NO	
Milk and products thereof (including lactose)	NO	
Nuts* and products thereof	NO	
Celery and products thereof	NO	
Mustard and products thereof	NO	
Sesame Seeds and products thereof	NO	
Lupine and products thereof	NO	
Molluscs and products thereof	NO	
Sulfur Dioxide and Sulfites at concentrations of more than 10 mg/kg or 10 mg/litre expressed as SO_2	NO	

*Please consult the EU Labelling Directive 2000/13 Annex IIIa for legal definition of common allergens, see European Union law at: http://eur-lex.europa.eu/

Chr. Hansen A/S -10-12 Bøge Allé - DK-2970 Hørsholm, Denmark - Phone: +45 45 74 74 74 - Fax: +45 45 74 88 88
Chr. Hansen, Inc. USA - 16300 W. Lincoln Ave. - New Berlin, WI 53151 USA - Phone: 888-289-2218 - Fax: 262-814-2188
www.chr-hansen.com

CHR. HANSEN

Statement

August 6, 2009

Valid two years from date of issue

To whom it may concern

Raw materials conformity

Thank you for your inquiry into Chr. Hansen's products.

All our raw materials for production of frozen and freeze-dried cultures are approved for use in food products.

All our vendors are approved before delivering ingredients to Chr. Hansen. Each vendor answers an electronic questionnaire, which include quality, food safety and CSR questions. Based on these answers and a risk assessment of the influence of the raw materials in our products, a vendor audit may be performed.

Our suppliers duly sign the specifications in order to ensure a mutual agreement on the quality, food safety and legal requirements applying to the product supplied to us.

The raw materials are inspected for correct delivered product, external damage or signs of non-conformity and inspected for possible contamination or signs of tampering and registered in SAP, Chr. Hansen's ERP system (Entreprise Ressource Planning system). All raw materials are registered in SAP, with full traceability to the supplier, date of receipt, batch no. etc.

Raw materials are set in quarantine upon arrival and have to be approved by the Quality Control Laboratory before being released for use in production. The approvals depend on the type of raw material and can be either analysis or a compliance check of the Certificate of analysis.

If you have further questions feel free to contact us.

Yours sincerely
Chr. Hansen A/S
Commercial Development, Food Cultures & Enzymes
Karoline Kjaerulff
Product Service Manager

Electronically generated, therefore not signed

Chr. Hansen A/S -10-12 Bøge Allé - DK-2970 Hørsholm, Denmark - Phone: +45 45 74 74 74 - Fax: +45 45 74 88 88 - www.chr-hansen.com

APPENDIX C

Appendix C

Summary of Human Studies

Table C-1 Oral Administration of *Lactobacillus reuteri* to Healthy Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Wolf *et al.*, 1995 Safety and tolerance of *Lactobacillus reuteri* in healthy adult male subjects	Randomized double-blind placebo controlled intervention	30 healthy male adults	*L. reuteri* MM53; ATCC SD2112 (5x1010 CFU/day *via* capsule) Placebo capsule (cryoprotectant alone)	28 days	• No between group differences in body weight; pulse rate; systolic and diastolic blood pressure; and body temperature • SS difference in respiratory rate between groups at day 28; placebo produced greater lowering of respiratory rate • Sporadic SS between group differences in clinical chemistry and hematology parameters noted throughout the study; however, all values remained within the expected normal range for healthy adult males • SS increase in urine indican in *L. reuteri* group at day 7 relative to placebo, but no between group difference at day 28 • No other between group difference in urinary parameters • SS increase in *L. reuteri* colonization throughout the study in the *L. reuteri* group; however, colonization was lost within 2 months • SS increase from baseline in total lactobacillus counts in the *L. reuteri* group relative to controls • No between group differences in fecal fat levels • Incidence of subjective gastrointestinal tolerance endpoints were infrequent and similar between treatment groups

Table C-1 Oral Administration of *Lactobacillus reuteri* to Healthy Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Johansson *et al.*, 1993 Administration of different *Lactobacillus* strains in fermented oatmeal soup: in vivo colonization of human intestinal mucosa and effect on the indigenous flora	Uncontrolled intervention	13 healthy subject (9 ♀ and 4 ♂), 31 to 56 years of age, with no recent antibiotic use	1. *L. salivarius* 132, *L. salivarius* 280, *L. reuteri* 108, *L. reuteri* R2LC, *L. casei pseudoplantarum* 136, *L. jenseni-L. gasseri* 140, *L. jenseni-L. gasseri* 292, *L. acidophilus-L. crispatus* 308, *L. plantarum* 283, *L. plantarum* 299, *L. plantarum* 299v, *L. casei rhamnosus* 98, *L. casei rhamnosus* 271, *L. agilis* 294, *Lactobacillus* sp. strain 96, *Lactobacillus* sp. strain 99, *Lactobacillus* sp. strain 138, *Lactobacillus* sp. strain 227, and *Lactobacillus* sp. strain 282. $5x10^8$ CFU/strain/day in fermented oatmeal soup	10 days	• Safety data not presented • SS increase in *Lactobacillus* counts on jejuna mucosa • SS decrease in anaerobic and gram-negative anaerobic bacteria counts in rectal mucosa • Colonization of jejunal and rectal mucosa variable
Guerrero *et al.*, 1996 Effect of probiotic-containing beverages on incidence of diarrhea (abstract)	Randomized, double-blind, placebo-controlled intervention	388 healthy children, 12 to 32 months of age	1. *L. reuteri* + other probiotics (strains/doses not reported) 2. Other probiotics (strains/doses not reported)	16 weeks	• SS lower incidence of diarrhea in *L. reuteri* group • Safety data not presented
Ruiz-Palacios *et al.*, 1996a Tolerance and fecal colonization with *Lactobacillus reuteri* in children fed a beverage with a mixture of *Lactobacillus* spp. (abstract)	Randomized, blinded, placebo-controlled intervention	72 children, 12 to 36 months of age	1. *L. reuteri*, *L. acidophilus*, and *B. infantis* ($1x10^6$ CFU/day) 2. *L. reuteri*, *L. acidophilus*, and *B. infantis* ($1x10^8$ CFU/day) 3. *L. reuteri*, *L. acidophilus*, and *B. infantis* ($1x10^{10}$ CFU/day)	3 weeks	• No significant between group differences in vomiting, abdominal discomfort, gas and stool characteristics • Intake of probiotic mixture well tolerated • *Lactobacillus* colonization is transient and fecal colonization is dose-related

Table C-1 Oral Administration of *Lactobacillus reuteri* to Healthy Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Jacobsen *et al.*, 1999 Screening of probiotic activities of 47 strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of 5 selected strains in humans	Randomized, double-blind, placebo-controlled, crossover intervention	12 healthy men, 18 to 37 years of age	1. *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246 2. *L. rhamnosus* LGG, *L. delbrueckii spp. Lactis* CHCC 2329, and *L. casei spp. alactis* CHCC 3137 All probiotics consumed at dose of $2x10^{10}$ CFU/day	18 days, with 17-day washout periods	• Safety data not presented • *L. rhamnosus* 19070-2, *L. reuteri* DSM 12246, and *L. rhamnosus* LGG identified most frequently in fecal samples
Reid *et al.*, 2001 Probiotic *Lactobacillus* dose required to restore and maintain a normal vaginal flora	Randomized controlled intervention	42 healthy women, 17 to 50 years of age	1. *L. rhamnosus* GR-1 and *L. fermentum* RC-14 ($4x10^8$ CFU each/day) 2. *L. rhamnosus* GR-1 and *L. fermentum* RC-14 ($8x10^9$ CFU each/day) 3. *L. rhamnosus* GR-1 and *L. fermentum* RC-14 ($3x10^9$ CFU each/day) 4. *L. rhamnosus* GG ($1x10^{10}$ CFU/day)	28 days	• No adverse events reported • Combined probiotic treatment associated with healthy vaginal flora in 90% of subjects • 7/11 subjects with BV showed improvement following consumption of combined probiotic treatment
Rosenfeldt *et al.*, 2003a Faecal recovery, mucosal adhesion, gastrointestinal effects, and tolerance of mixed cultures of potential probiotic *Lactobacilli*	Randomized, double-blind crossover intervention	13 healthy subjects (♂); 17 to 29 years of age	1. *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246 ($2x10^{10}$ CFU each/day) 2. *L. casei alactus* CHCC 3137, *L. delbrueckii lactis* CHCC 2329, and *Lactobacillus* GG ATCC 53103 ($2x10^{10}$ CFU each/day)	18 days (with 17-day washout periods)	• Treatment was well tolerated and without adverse effects • 2 subjects reported mild, transient abdominal pain during consumption of *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246, and 1 reported abdominal pain and loose stools during consumption of placebo • SS decrease in fecal total *staphylococci* and *S. aureus* levels following consumption of *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246 • No effect of treatment on

Table C-1 Oral Administration of *Lactobacillus reuteri* to Healthy Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
					defecation frequency, stool consistency, gastrointestinal transit time, serum lipids, or fecal anaerobic, aerobic, or *Lactobacillus* cultures
Rosenfeldt *et al.*, 2003a Faecal recovery, mucosal adhesion, gastrointestinal effects, and tolerance of mixed cultures of potential probiotic *Lactobacilli*	Uncontrolled intervention	11 adults; 33 to 68 years of age; all having undergone removal of benign colonic polyps or with family history of polyposis	1. *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246 ($2x10^{10}$ CFU each/day)	10 days	• No adverse events reported • Fecal recovery of probiotics dependent on location of colonic biopsy (recovery highest in proximal regions)
Reid *et al.*, 2003a Oral use of *Lactobacillus rhamnosus* gr-1 and *L. fermentum* RC-14 significantly alters vaginal flora: randomized, placebo-controlled trial in 64 healthy women	Randomized, double-blind, placebo-controlled intervention	64 healthy women, 19 to 46 years of age, with no history of urogenital infection (within 12 months of study start date)	1. *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (>$1x10^{9}$ CFU each/day)	60 days	• No adverse events reported • SS more women in probiotic group showed restoration of normal vaginal flora (*vs.* placebo) • SS more *Lactobacilli*, and fewer yeasts and coliforms, detected in probiotic group (*vs.* placebo)
Nikawa *et al.*, 2004 *Lactobacillus reuteri* in fermented bovine milk decreases the oral carriage of mutans streptococci	Placebo-controlled crossover intervention	40 subjects with healthy mouths	1. *L. reuteri* (dose/strain not reported)	2 weeks	• Safety data not presented

Table C-1 Oral Administration of *Lactobacillus reuteri* to Healthy Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Valeur *et al.*, 2004 Colonization and immunomodulation by *Lactobacillus reuteri* ATCC 55730 in the human gastrointestinal tract	Open-label, single-arm crossover intervention	10 healthy adults and 9 adults with ileostomy (following colectomy for ulcerative colitis or Crohn's disease); >18 years of age	1. *L. reuteri* ATCC 55730 ($4x10^8$ CFU/day)	28 days	• No evidence of damage to gastric or intestinal mucosa • No adverse events reported, except for SS higher flatulence scores in healthy subjects • *L. reuteri* detected in feces of all healthy subjects and 6/9 ileostomy subjects on day 28 • SS increased number of B lymphocytes in duodenal biopsy specimens • SS reduction in number of CD68-positive cells in the corpus and antrum epithelia • SS increase in CD4-positive T lymphocytes in ileal epithelium
Morelli *et al.*, 2004 Utilization of the intestinal tract as a delivery system for urogenital probiotics	Randomized, double-blind, placebo-controlled intervention	10 healthy women with no history of urogenital infection (within 12 months of study start date)	1. *L. rhamnosus* GR-1 and *L. fermentum* RC-14 (dose not reported)	14 days	• Safety data not presented • *L. rhamnosus* GR-1 identified in feces and vaginal swabs of 8/8 and 5/8 subjects in probiotic group (respectively) • *L. fermentum* RC-14 identified in feces and vaginal swabs of 4/8 and 2/8 subjects in probiotic group (respectively)
Jakobsson *et al.*, 2005 The effect of oral supplementation of *Lactobacillus reuteri* on the immunological composition of breast milk (abstract)	Placebo-controlled intervention [ABSTRACT ONLY]	109 pregnant women	1. *L. reuteri* ATCC 55730 ($1x10^8$ CFU/day)	4 weeks (last 4 weeks of pregnancy)	• Safety data not presented • SS higher IL-10 levels and lower TGF-β2 levels in colostrum in *L. reuteri* group (*vs.* placebo)

Table C-1 Oral Administration of *Lactobacillus reuteri* to Healthy Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Tubelius *et al.*, 2005 Increasing work-place healthiness with the probiotic *Lactobacillus reuteri*: a randomised, double-blind placebo-controlled study	Randomized, double-blind, placebo-controlled intervention	181 healthy adults (136 ♂, 64 ♀); mean age: 44 years	1. *L. reuteri* ATCC 55730 ($1x10^8$ CFU/day; consumed *via* drinking straw)	80 days	• SS fewer sick leaves (related to respiratory/GI illness) and lower frequency of sick days in probiotic group (*vs.* placebo) • No adverse events reported
Tubelius *et al.*, 2005 Increasing work-place healthiness with the probiotic *Lactobacillus reuteri*: a randomized, double-blind placebo-controlled study	Randomized double-blind placebo controlled intervention	262 healthy adults (136♂ and 64 ♀); mean age 44 years	*L. reuteri* ATCC55730 (10^8 CFU/day *via* probiotic straw)	80 days	• No adverse events reported during the study • SS reduction in sick days in *L. reuteri* group
Krasse *et al.*, 2006 Decreased gum bleeding and reduced gingivitis by the probiotic *Lactobacillus reuteri*	Randomized, double-blind, placebo-controlled intervention	59 adults with moderate to severe gingivitis	1. *L. reuteri* LR-1 2. *L. reuteri* LR-2 Both probiotics consumed as chewing gum (doses not reported)	14 days	• Safety data not presented
Caglar *et al.*, 2006 Salivary mutans streptococci and lactobacilli levels after ingestion of the probiotic bacterium *Lactobacillus reuteri* ATCC 55730 by straws or tablets	Randomized, double-blind, placebo-controlled intervention	120 healthy adults (71 ♂, 49 ♀), 21 to 24 years of age	1. *L. reuteri* ATCC 57730 ($1x10^8$ CFU/day, consumed *via* a straw containing probiotic used to drink 200 mL water) 2. *L. reuteri* ATCC 57730 ($1x10^8$ CFU/day, consumed *via* lozenge)	3 weeks	• Safety data not presented • SS reduction in salivary *S. mutans* levels in both probiotic groups (*vs.* placebo) • NS change in salivary *Lactobacilli* levels

Table C-1 Oral Administration of *Lactobacillus reuteri* to Healthy Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Boyle *et al.*, 2006 Probiotic use in clinical practice: what are the risks?	Review	>36 individuals, <1 to 89 years of age	All strains not identified, but included: *Lactobacillus* GG, *Bacillus subtilis*, and *Saccharomyses boulardii*	Not reported	• Safety assessment: probiotics generally safe for use in otherwise healthy populations • Reported adverse events included: liver abscess, endocarditis, bacteremia, fungemia, septic shock, septicemia, central venous catheter colonization, fungemic shock, and fatal fungemia • Risk factors for probiotic sepsis: immune compromise (including debilitated state or malignancy), premature infants, presence of a central venous catheter, impaired intestinal epithelial barrier, administration of probiotic by jejunostomy, concomitant administration of broad-spectrum antibiotic to which probiotic is resistant, probiotics with high mucosal adhesion or known pathogenicity, and cardiac valvular disease (*Lactobacilli* only)
Weizman and Alsheik, 2006 Safety and tolerance of a probiotic formula in early infancy comparing two probiotic agents: a pilot study	Randomized, double-blind, placebo-controlled intervention	59 children, 3 to 65 days of age	1. *B. lactis* Bb12 ($1.2x10^9$ CFU/day) 2. *L. reuteri* ($1.2x10^9$ CFU/day)	4 weeks	• No adverse events reported • No differences between probiotic groups or placebo group with respect to growth, feeding, behavior, or stooling

Table C-1 Oral Administration of *Lactobacillus reuteri* to Healthy Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Caglar *et al.*, 2007 Effect of chewing gums containing xylitol or probiotic bacteria on salivary mutans streptococci and lactobacilli	Randomized, double-blind, placebo-controlled intervention	80 healthy adults (36 ♂, 44 ♀), 21 to 24 years of age	1. *L. reuteri* ATCC 57730 ($3x10^8$ CFU/day) and *L. reuteri* ATCC PTA 5289 ($3x10^8$ CFU/day), consumed as chewing gum 2. 1.03 g xylitol, consumed as chewing gum 3. 1.03 g xylitol, *L. reuteri* ATCC 57730 ($3x10^8$ CFU/day), and *L. reuteri* ATCC PTA 5289 ($3x10^8$ CFU/day), consumed as chewing gum	3 weeks	• Safety data not presented • SS reduction in salivary *S. mutans* levels in probiotic and xylitol groups (*vs.* baseline) • No change in salivary *Lactobacilli* in any group
Böttcher *et al.*, 2008 Low breast milk TGF-β2 is induced by *Lactobacillus reuteri* supplementation and associates with reduced risk of sensitization during infancy	Randomized, double-blind, placebo-controlled intervention	109 pregnant women (21 to 44 years of age) and their newborn children	1. *L. reuteri* ATCC 55730 (dose not reported)	Mothers: week 36 of gestation until delivery Children: from birth to 12 months of age	• Safety data not presented • *L. reuteri* consumption associated with low levels of TGF-β in breast milk • Infants consuming breast milk with low levels of TGF-β less likely to become sensitized in first 2 years of life or to develop IgE-associated eczema
Caglar *et al.*, 2008 A probiotic lozenge administered medical device and its effect on salivary mutans streptococci and lactobacilli	Randomized, double-blind, placebo-controlled intervention	20 healthy adult women, approximately 20 years of age, with high levels of salivary S. mutans (≥105 CFU)	1. *L. reuteri* ATCC 57730 and *L. reuteri* ATCC PTA 5289 ($1.1x10^8$ CFU/day, in a 10:1 ratio), consumed *via* lozenge in slow-release medical device (pacifier)	10 days	• Safety data not presented • SS reduction in salivary *S. mutans* levels in probiotic group (*vs.* placebo)
Twetman *et al.*, 2009 Short-term effect of chewing gums containing probiotic *Lactobacillus reuteri* on the levels of inflammatory mediators in gingival crevicular fluid	Randomized, double-blind, placebo-controlled intervention	42 adults with moderate gingivitis	1. *L. reuteri* ATCC 55730 and *L. reuteri* ATCC PTA 5289 ($1x10^8$ CFU each/day) 2. *L. reuteri* ATCC 55730 and *L. reuteri* ATCC PTA 5289 ($2x10^8$ CFU each/day)	2 weeks	• Safety data not presented

Table C-1 Oral Administration of *Lactobacillus reuteri* to Healthy Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Rosander *et al.*, 2008 Removal of antibiotic resistance plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain *L. reuteri* DSM 17938	Randomized, double-blind, placebo-controlled intervention	16 healthy adults	1. *L. reuteri* ATCC 55730 ($8x10^8$ CFU/day) 2. *L. reuteri* DSM 17938 ($8x10^8$ CFU/day) 3. *L. reuteri* DSM 17938 ($6.5x10^{10}$ CFU/day)	28 days	• No changes in weight, pulse, blood pressure, or body temperature during intervention period • No changes in blood safety (major blood components and liver, kidney, and immune function tests) and metabolic parameters • All blood samples taken on day 28 were negative for bacteremia • GI survival was similar in both strains
Ojetti *et al.*, 2010 The effect of oral supplementation with *Lactobacillus reuteri* or tilactase in lactose-intolerant patients: a placebo controlled study	Randomized, placebo-controlled intervention	60 healthy lactose-intolerant subjects (6 ♂, 54 ♀), 18 to 65 years of age	1. *L. reuteri* ($8x10^8$ CFU/day) 2. Tilastase (9000 U, taken 15 minutes prior to breath hydrogen test)	10 days (preceding breath hydrogen testing)	• No "relevant" adverse events reported (2 mild cases of diarrhea and 1 mild case of constipation in *L. reuteri* group) • SS reductions form baseline in mean clinical score, bloating, abdominal pain, flatulence, and diarrhea in *L. reuteri* group • SS reduction in breath H_2 concentration in *L. reuteri* group (*vs.* placebo) • SS improvement in symptoms and breath H_2 concentration in Tilactase group (*vs.* placebo and *L. reuteri* groups)

Table C-2 Oral Administration of *L. reuteri* to Unhealthy or Diseased Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Ruiz-Palacios *et al.*, 1996a Tolerance and fecal colonization with *Lactobacillus reuteri* in children fed a beverage with a mixture of lactobacillus spp. (abstract)	Randomized blinded placebo controlled intervention	72 Children ages 12 to 36 months	Four groups in total *L. reuteri*, *L. acidophilus*, *B. infantis* in liquid nutritional beverage (0, 10^6, 10^8, or 10^{10} CFU/beverage)	3 weeks	• No significant between group differences in vomiting, abdominal discomfort, gas and stool characteristics • Intake of probiotic mixture well tolerated • Lactobacillus colonization is transient and fecal colonization is dose-related
Ruiz-Palacios *et al.*, 1996b Feeding a probiotic for the prevention of community-acquired diarrhea in young Mexican children (abstract)	Randomized, blinded, placebo-controlled intervention	243 children	1. *L. reuteri* probiotic beverage	14 weeks	• SS reduction in incidence of diarrhea in the *L. reuteri* group • No difference in severity of diarrhea between groups • Reduced incidence of rotavirus diarrhea in the *L. reuteri* group
Ruiz-Palacios *et al.*, 1996b (abstract) Feeding a probiotic for the prevention of community-acquired diarrhea in young Mexican children	Randomized blinded placebo controlled intervention	243	*L. reuteri* probiotic beverage	14 weeks	• SS reduction in incidence of diarrhea in the *L. reuteri* group • No difference in severity of diarrhea between groups • Reduced incidence of rotavirus diarrhea in the *L. reuteri* group

Table C-2 Oral Administration of *L. reuteri* to Unhealthy or Diseased Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Shornikova *et al.*, 1997a *Lactobacillus reuteri* as a therapeutic agent in acute diarrhea in young children	Randomized, double-blind, controlled intervention	40 healthy, well-nourished, immunocompetent children (~61% ♂); 6 to 36 months of age; all hospitalized due to acute diarrhea of <7 days in duration (due to rotavirus in 63% of treatment and 86% of placebo subjects)	1. *L. reuteri* SD2112 ($1x10^{10}$ to $1x10^{11}$ CFU/day) All subjects received standard treatment (oral rehydration) for diarrhea, without antidiarrheal drugs	6 days, or the duration of hospitalization, whichever was shorter	• Safety data not presented • Slight decrease in duration of watery diarrhea in *L. reuteri* group • SS reduction in mean frequency of watery diarrhea, number of patients with water diarrhea, and number of patients with vomiting in *L. reuteri* group • Greater amount of *Lactobacilli* in feces of *L. reuteri* group • Increased fecal urease levels in placebo group, but not in *L. reuteri* group
Shornikova *et al.*, 1997b Bacteriotherapy with *Lactobacillus reuteri* in rotavirus gastroenteritis	Randomized, double-blind, controlled intervention	97 children; 6 to 36 months of age; all hospitalized due to acute diarrhea of <7 days in duration (due to rotavirus in 89% of cases)	1. *L. reuteri* ($1x10^{10}$ to $1x10^{11}$ CFU/day) 2. *L. reuteri* ($1x10^{7}$ CFU/day with 0.5 g lactose) All subjects received standard treatment (oral rehydration) for diarrhea, without antidiarrheal drugs	6 days, or the duration of hospitalization, whichever was shorter	• Safety data not presented • SS reduction of number of subjects with diarrhea, duration of diarrhea, and frequency of watery diarrhea in *L. reuteri* groups *vs.* placebo • Increased total *Lactobacilli* and *L. reuteri* counts in feces of treated subjects (*vs.* placebo) • Increased urease activity in placebo group; decreased urease activity in *L. reuteri* groups
Wolf *et al.*, 1998 Safety and tolerance of *Lactobacillus reuteri* supplementation to a population infected with the human immunodeficiency virus	Randomized, double-blind, placebo-controlled intervention	39 (37 ♂ and 2 ♀) adults with HIV; 23 to 50 years of age	1. *L. reuteri* SD2112 ($1x10^{10}$ CFU/day)	21 days	• Overall tolerance was good in both groups • No clinically significant changes noted in any safety parameters assessed (serum chemistry, hematology, immune deficiency, and urinalysis) • SS increases from baseline in diastolic blood pressure and urinary specific gravity in *L. reuteri vs.* placebo group

Table C-2 Oral Administration of *L. reuteri* to Unhealthy or Diseased Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
					• NS trend towards more complaints of mild nausea in *L. reuteri vs.* placebo group
Björkman, 1999 Colonization of the human gastrointestinal tract by the lactic acid bacteria *Lactobacillus reuteri* (English abstract only – Finnish)	Open-label intervention	Adults undergoing colonoscopy examination	1. *L. reuteri* ($1x10^9$ CFU/day; consumed as yogurt) 2. *L. reuteri* ($1x10^9$ CFU/day; consumed as fruit juice)	12 days	• Safety data not presented
Saggioro *et al.*, 2005 Helicobacter pylori eradication with *Lactobacillus reuteri*, a double-blind placebo controlled study (abstract)	Randomized double-blind placebo controlled intervention	30 Patients with dyspepsia and positive to urea breath test Aged 25 to 56	*L. reuteri* ($1.6x10^6$ CFU/day) + omeprazole (40 mg/day) Placebo + omeprazole	30 days	• SS increase in eradication of *H. pylori* infection in the *L. reuteri* group relative to the placebo group
Rosenfeldt *et al.*, 2002a Effect of probiotic Lactobacillus strains in young children hospitalized with acute diarrhea	Randomized, double-blind, placebo-controlled intervention	69 children hospitalized for acute diarrhea (30 ♀, 39 ♂), 6 to 36 months of age	1. *L. rhamnosus* 19070-2 ($3.4x10^{10}$ CFU/day) and *L. reuteri* DSM 12246 ($1x10^{10}$ CFU/day)	5 days	• Safety data not presented • SS fewer subjects with loose stools or fecal rotavirus antigen on day 5 in probiotic group (*vs.* placebo) • SS reduced length of hospitalization and duration of diarrhea in subjects with diarrhea for <60 hours in probiotic group

Table C-2 Oral Administration of *L. reuteri* to Unhealthy or Diseased Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Rosenfeldt *et al.*, 2002b Effect of probiotic lactobacillus strains on acute diarrhea in a cohort of nonhospitalized children attending day-care centers	Randomized, double-blinded, placebo-controlled intervention	43 children with acute diarrhea (17♂ and 26♀), mean age: 22.5 and 21.1 months (treatment and placebo group)	1. *L. reuteri* SDM 12246 and *L. rhamnosus* 19070-2 ($2x10^{10}$ CFU each/day)	5 days	• No serious adverse events • On subject in treatment group complained of constipation on day 3 of intervention and for a further 10 days • SS reduction in duration of diarrhea in probiotic group • One or both strains recovered from feces of 65% of subjects examined
Rosenfeldt *et al.*, 2002b Effect of probiotic lactobacillus strains on acute diarrhea in a cohort of nonhospitalized children attending day-care centers	Randomized double blinded placebo controlled intervention	43 Children with acute diarrhea (17♂ and 26♀) Mean age of 22.5 and 21.1 months in treatment and placebo groups	*L. reuteri* SDM 12246 and *L. rhamnosus* 19070-2 ($2x10^{10}$ CFU/day of each strain *via* lyophilized powder)	5 days	• No serious adverse events • On subject in treatment group complained of constipation on day 3 of intervention and for a further 10 days • SS reduction in duration of diarrhea in probiotic group • One or both strains recovered from feces of 65% of subjects examined
Ouwehand *et al.*, 2002 Effect of probiotics on constipation, fecal azoreductase activity, and fecal mucin content in the elderly	Open-label parallel intervention	28 elderly subjects (7 ♂ and 21 ♀) with difficulties in defecation, all living in a nursing home	1. Bioprofit (orange juice with 1 to $2x10^8$ CFU/day *L. rhamnosus* LC705 and 2 to $4x10^8$ CFU *Propionibacterium freudenreichii shermanii* JS/day) 2. Rela (orange juice with $3.6x10^6$ CFU *L. reuteri* ING1/day)	4 weeks	• Safety data not presented • SS increased defecation frequency in Rela group (*vs.* orange juice control) during intervention period (but not *vs.* baseline) • SS increase in defecation frequency and reduction in fecal azoreductase activity in Bioprofit group (*vs.* baseline)

Table C-2 Oral Administration of *L. reuteri* to Unhealthy or Diseased Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Reid *et al.*, 2003b Effect of Lactobacilli oral supplement on the vaginal microflora of antibiotic treated patients: randomized, placebo-controlled study	Randomized, double-blind, placebo-controlled intervention	24 adults females with oral, throat, or respiratory infections requiring antibiotics	1. *L. rhamnosus* GR-1 and *L. fermentum* RC-14 (combined dose: >2x10^9 CFU/day)	21 days (beginning on same day as antibiotic treatment)	• No adverse events reported (including diarrhea or yeast vaginitis) • Lower BV symptom scores in probiotic group (*vs.* placebo) • No incidences of BV in probiotic group (*vs.* 3 cases in placebo group)
Rosenfeldt *et al.*, 2003b Effect of probiotic lactobacillus strains in children with atopic dermatitis	Randomized, double-blind, placebo-controlled, crossover intervention	45 children with atopic dermatitis (42% ♂), 1 to 13 years of age	1. *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460 (2x10^{10} CFU each/day)	6 weeks, with a 6-week washout period	• 1 subject withdrawn due to exacerbation of eczema requiring hospitalization (in placebo group) • 1 subject withdrawn due to exacerbation of eczema requiring systemic corticosteroids (in probiotic group) • No adverse events in remaining study population reported • SS more improvement in eczema and inflammatory cytokine levels in probiotic group (*vs.* placebo) • Improvement in eczema more pronounced in allergic subjects (with positive SPT and elevated IgE levels)
Rosenfeldt *et al.*, 2004 Effect of probiotics on gastrointestinal symptoms and small intestinal permeability in children with atopic dermatitis	Randomized, double-blind, placebo-controlled crossover intervention	41 children with moderate to severe atopic dermatitis, 1 to 13 years of age	1. *L. rhamnosus* 19070-2 and *L. reuteri* DSM 12246 (2x10^{10} CFU each/day)	6 weeks	• GI symptoms during intervention: vomiting (1 in placebo group); diarrhea (6 in placebo group, 1 in probiotic group); abdominal pain (6 in placebo group, 2 in probiotic group); diarrhea and abdominal pain (3 in placebo group, 1 in probiotic group); any GI symptom (6 in placebo group, 4

Table C-2 Oral Administration of *L. reuteri* to Unhealthy or Diseased Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
					in probiotic group) • No episodes of acute gastroenteritis reported • SS decrease in frequency of GI symptoms in probiotic group (*vs.* placebo) • SS reduction from baseline in urinary lactulose:mannitol in probiotic group
Eom *et al.*, 2005 The therapeutic effect of *Lactobacillus reuteri* in acute diarrhea in infants and toddlers	Randomized, placebo-controlled intervention	50 children, 3 to 36 months of age, hospitalized with acute diarrhea	1. *L. reuteri* ($2x10^8$ CFU/day)	Length of hospital stay (up to 5 days)	• SS reductions in watery diarrhea, frequency of diarrhea, and vomiting (*vs.* placebo) • Safety data not presented
Cirillo *et al.*, 2005 Effectiveness of *L. reuteri* in patients with atopic dermatitis and cow milk intolerance: preliminary study (abstract)	Open-label intervention	15 children, 3 to 5 years of age, with mild atopic dermatitis aggravated by intake of cow's milk	1. *L. reuteri* ($2x10^8$ CFU/day) All subjects orally challenged with cow's milk during entire intervention period	3 months	• Reduced aggravation of dermatitis in *L. reuteri* group (*vs.* placebo) • Safety data not presented
Niv *et al.*, 2005 The efficacy of *Lactobacillus reuteri* ATCC 55730 in the treatment of patients with irritable bowel syndrome – a double blind, placebo-controlled, randomized study	Randomized, double-blind, placebo-controlled intervention	54 otherwise healthy and non-pregnant adults with IBS (scoring ≥75/500 on the Francis Severity IBS score); 19 to 70 years of age; 66.7% ♀ 39 subjects completed the study (per-protocol population)	1. *L. reuteri* ATCC 55730 ($2x10^8$ CFU /day)	6 months	• No significant between-group differences in compliance, occurrence of adverse events, or changes in the severity score or the different components of the total quality of life score

Table C-2 Oral Administration of *L. reuteri* to Unhealthy or Diseased Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Lionetti *et al.*, 2006 *Lactobacillus reuteri* therapy to reduce side-effects during anti-helicobacter pylori treatment in children: a randomized placebo controlled trial	Randomized, double-blind, placebo-controlled intervention	40 H. pylori-positive children (21 ♂, 19 ♀), 3 to 18 years of age	1. *L. reuteri* ATCC 55730 ($1x10^8$ CFU/day) All subjects received 15 days of antibiotic therapy	20 days	• No adverse events reported • *L. reuteri* group reported fewer overall symptoms than placebo group during and after antibiotic therapy for *H. pylori* infection
Anukam *et al.*, 2006 Augmentation of antimicrobial metronidazole therapy of bacterial vaginosis with oral probiotic *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14: randomized, double-blind, placebo controlled trial	Randomized, double-blind, placebo-controlled intervention	125 women (18 to 44 years of age) with BV (all receiving oral metronidazole)	1. *L. reuteri* RC-14 ($2x10^9$ CFU/day) and *L. rhamnosus* GR-1 ($2x10^9$ CFU/day)	30 days	• 19 withdrawals (16 in probiotic group; reasons not reported) • No adverse events reported • 2 subjects in probiotic group reported persistent headaches (resolved after 3 days of treatment) and increased appetite (resolved after 5 days of treatment) • SS higher number of subjects with BV resolution in probiotic group (*vs.* placebo) • SS higher vaginal *Lactobacillus* counts in probiotic group (*vs.* placebo)
Lorea Baroja *et al.*, 2007 Anti-inflammatory effects of probiotic yogurt in inflammatory bowel disease patients	Uncontrolled, single-blind intervention	20 subjects with IBD (15 with CD and 5 with UC) and 20 healthy controls, 26 to 63 years of age	1. *L. rhamnosus* GR-1 ($2x10^7$ CFU/mL yogurt) and *L. reuteri* RC-14 ($1x10^3$ CFU/mL yogurt)	30 days	• SS increase from baseline in reported flatulence and "low" abdominal pain in subjects with IBD • Increased proportion of $CD4+CD25^{high}$ T cells; decrease in TNF-α, IL-12, IL-2, CD69+ T cells and myeloid DC in IBD subjects (suggestive of anti-inflammatory effects)

Table C-2 Oral Administration of *L. reuteri* to Unhealthy or Diseased Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Abrahamsson *et al.*, 2007 Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial	Randomized, double-blind, placebo-controlled intervention	227 families (with maternal or paternal history of eczema, asthma, gastrointestinal allergy, allergic urticaria, or allergic rhinoconjunctivitis) and their newborn children (n=188)	1. *L. reuteri* ATCC 55730 ($1x10^8$ CFU/day)	Mothers: product taken from 4 weeks before term until delivery Children: product taken from birth to 12 months of age	• 19 children were withdrawn from the study between 1 week and 12 months of age; no reason was given for 16 withdrawals, and abdominal discomfort/colic was given as the reason for 3 (2 in *L. reuteri* group) • 1 infant withdrawn from the *L. reuteri* group had an episode of wheezing at 2 months of age – cumulative incidence of wheezing similar between intervention and placebo groups • No significant difference in cumulative incidence of mild adverse events (spitting up, colic, and constipation) during first 12 months • No severe adverse events reported • SS higher incidence of spitting up in *L. reuteri* group at 1 and 2 months of age, although no differences in reported gastrointestinal disturbances were observed • SS higher incidence of antibiotics prescription (mostly for otitis media) during first 12 months in *L. reuteri* group (adjustment for antibiotic treatment did not change results)

Table C-2 Oral Administration of *L. reuteri* to Unhealthy or Diseased Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Imase *et al.*, 2007 *Lactobacillus reuteri* tablets suppress *Helicobacter pylori* infection--a double-blind randomised placebo-controlled cross-over clinical study	Randomized, double-blind, placebo-controlled, crossover intervention	35 H. pylori-positive and 5 H. pylori-negative patients (19 ♂, 21 ♀), mean age: 47.8 years	1. *L. reuteri* 55730 ($1x10^8$ CFU/day)	4 to 8 weeks	• Safety data not presented • SS lower exhaled urea levels (associated with *H. pylori* activity and density) following consumption of *L. reuteri* (*vs.* placebo)
Anukam *et al.*, 2008 Yogurt containing probiotic *Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14 helps resolve moderate diarrhea and increases cd4 count in HIV/AIDS patients	Randomized, double-blind, placebo-controlled intervention	24 lactose-tolerant, premenopausal, adult females with HIV/AIDS, and clinical moderate diarrhea, CD4 counts >200, and not receiving dietary supplements or antiretroviral treatment	1. *L. reuteri* RC-14 ($1x10^9$ CFU/day) and *L. rhamnosus* GR-1 ($1x10^9$ CFU/day) in conventional yogurt	15 days	• No deaths occurred during the intervention or 3-month follow-up periods • 3 subjects in placebo group developed skin rashes (none reported in probiotic group) • Rapid resolution of diarrhea, flatulence, and nausea in probiotic group (compared to placebo group)
Francavilla *et al.*, 2008 Inhibition of Heliobacter pylori infection in humans by *Lactobacillus reuteri* ATCC 55730 and effect on eradication therapy: a pilot study	Randomized, double-blind, placebo-controlled intervention	40 dyspeptic adults infected with H. pylori	1. *L. reuteri* ATCC 55730 ($1x10^8$ CFU/day)	28 days	• No adverse events reported • Reduced H. pylori levels and GI symptoms in *L. reuteri* group (*vs.* placebo)
Chmielewska *et al.*, 2008 *Lactobacillus reuteri* strain ATCC 55730 for the treatment of acute infectious diarrhoea in children: a meta-analysis of randomized controlled trials	Meta-analysis of randomized controlled trials	106 children, 3 to 36 months of age, with acute infectious diarrhea (in 2 randomized controlled interventions)	1. *L. reuteri* ATCC 55730 ($1x10^7$ to $1x10^{11}$ CFU/day)	Up to 5 days	• Neither study reported adverse events • Both studies reported reductions in frequency and duration of diarrhea

Table C-2 Oral Administration of *L. reuteri* to Unhealthy or Diseased Adults and Children

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Martinez *et al.*, 2009 Improved treatment of vulvovaginal candidiasis with fluconazole plus probiotic Lactobacillus rhamnosus gr-1 and *Lactobacillus reuteri* rc-14	Randomized, double-blind, placebo-controlled intervention	55 women with symptomatic VVC, 16 to 46 years of age, all receiving fluconazole treatment	1. *L. rhamnosus* GR-1 and *L. reuteri* RC-14 ($2x10^9$ CFU each/day)	4 weeks	• 2 subjects in the probiotics group reported increased appetite • 1 subject in the probiotics group reported a single episode of headache • 1 subject in the probiotics group reported lighter stool • No reported adverse events could be attributed to probiotic consumption • SS fewer VVC-associated symptoms and yeast levels in probiotic group (*vs.* placebo)
Bruni *et al.*, 2009 Cow's milk allergic children can present sensitization to probiotic products	Crossover intervention	36 children with atopic dermatitis and positive SPT to cow's milk	1. Fiorilac (*L. paracasei* I 1688 and *L. salivarius* I 1794; dose not reported) 2. Dicoflor (*L. rhamnosus* GG; dose not reported) 3. Reuterin (*L. reuteri* protectis; dose not reported)		• 26/36 subject had positive SPT for Fiorilac • 2/36 subjects had positive SPT for Dicoflor • 1/36 subjects had positive SPT for Reuterin • No child with positive SPT for Dicoflor or Reuterin had mean wheal diameter >6 mm

Table C-3 Oral Administration of *Lactobacillus reuteri* to Healthy Infants

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Karvonen *et al.*, 2001 Safety and possible antidiarrhoeal effect of the probiotic *Lactobacillus reuteri* after oral administration to neonates (abstract)	Randomized, double-blind, placebo-controlled intervention	90 healthy, newborn, full-term children	1. *L. reuteri* ($1x10^5$ CFU/day) 2. *L. reuteri* ($1x10^7$ CFU/day) 3. *L. reuteri* ($1x10^9$ CFU/day)	From birth to 28 days of age	• *L. reuteri* was "well tolerated"
Asli *et al.*, 2003 Infant formula supplemented with probiotics affects morbidity in day care infants (abstract)	Randomized, double-blind, placebo-controlled intervention	194 healthy, full-term infants, 4 to 10 months of age	1. *L. reuteri* (dose/strain not reported) 2. *B. bifidum* (dose/strain not reported)	12 weeks	• SS fewer febrile episodes and incidences of GI illness in infants fed probiotic formulas (*vs.* control formula) • No adverse events reported
Abrahamsson *et al.*, 2005 Intestinal microbiota in infants supplemented with the probiotic bacterium *Lactobacillus reuteri* (abstract)	Randomized, placebo-controlled intervention	232 pregnant women	1. *L. reuteri* ($1x10^8$ CFU/day)	Mothers: 4 weeks (last 4 weeks of pregnancy) Children: from birth to 12 months of age	• Safety data not presented
Weizman *et al.*, 2005 Effect of probiotic infant formula on infections in child care centers: comparison of 2 probiotic agents	Randomized double-blind placebo controlled intervention	201 Healthy term infants 4 to 10 months old (96♂ and 105♀)	*L. reuteri* ATCC 55730 B. lactis BB-12 ($1x10^7$ CFU/g of infant formula powder for both treatment groups)	12 weeks	• No drop-outs due to study formula or adverse events • Adverse events were not noticed in any of the participants • No SS difference in growth parameters (*i.e.*, weight, length, and head circumference) • No reports of blood stools and no between group differences in stool parameters and infant behavior • No differences between groups in incidence of fecal pathogens

Table C-3 Oral Administration of *Lactobacillus reuteri* to Healthy Infants

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
					• No significant between group differences in number of daily meals, regurgitation and vomiting or infant compliance • Subjects in *L. reuteri* group reported SS fewer episodes of fever and diarrhea • SS reductions in clinic visits, absences from child care and prescriptions for antibiotics in *L. reuteri* group relative to control • No hospitalizations due to diarrhea • Incidence of indications requiring antibiotic therapy were otitis media, pneumonia, and upper respiratory infection did not differ between groups
Weizman *et al.*, 2005 Effect of probiotic infant formula on infections in child care centers: comparison of 2 probiotic agents	Randomized double-blind placebo controlled intervention	201 healthy term infants, 4 to 10 months of age (96♂ and 105♀)	1. *L. reuteri* ATCC 55730 2. *B. lactis* BB-12 ($1x10^7$ CFU/g of infant formula powder for both groups)	12 weeks	• No drop-outs due to study formula or adverse events • Adverse events were not noticed in any of the participants • No SS difference in growth parameters (*i.e.*, weight, length, and head circumference) • No reports of blood stools and no between group differences in stool parameters and infant behavior • No differences between groups in incidence of fecal pathogens • No significant between group differences in number of daily meals, regurgitation and vomiting or infant compliance

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Table C-3 Oral Administration of *Lactobacillus reuteri* to Healthy Infants

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
					• Subjects in *L. reuteri* group reported SS fewer episodes of fever and diarrhea • SS reductions in clinic visits, absences from child care and prescriptions for antibiotics in *L. reuteri* group relative to control • No hospitalizations due to diarrhea • Incidence of indications requiring antibiotic therapy were otitis media, pneumonia, and upper respiratory infection did not differ between groups
Indrio *et al.*, 2008 The effects of probiotics on feeding tolerance, bowel habits, and gastrointestinal motility in preterm newborns	Randomized, double-blind, placebo-controlled, crossover intervention	30 preterm newborns	1. *L. reuteri* ATCC 55730 ($1x10^8$ CFU/day)	30 days	• No adverse events reported • SS decrease in vomiting, mean daily crying time, and fasting antral area in probiotic group (*vs.* placebo) • SS increase in mean daily number of stools and gastric emptying rate in probiotic group (*vs.* placebo)
Indrio *et al.*, 2009 Effects of probiotic and prebiotic on gastrointestinal motility in newborns	Randomized, double-blind, placebo-controlled, crossover intervention	49 preterm newborns	1. Formula with galacto- and fructo- oligosaccharides (in a 9:1 ratio; 0.8 g/dL) 2. Formula with *L. reuteri* ($1x10^8$ CFU/day)	30 days	• No adverse events reported • SS higher GI motility in infants fed breast milk, prebiotics, and *L. reuteri* (*vs.* placebo)

Table C-4 Oral Administration of *Lactobacillus reuteri* to Unhealthy or Diseased Infants

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Connolly *et al.*, 2005 Safety of D(-)-lactic acid producing bacteria in the human infant	Randomized, double-blind, placebo-controlled intervention	Pregnant women from families with a history of allergic disease (eczema, asthma, gastrointestinal allergy, allergic urticaria, or allergic rhinoconjunctivitis) and their newborn children (n=24)	1. *L. reuteri* ATCC 55730 ($1x10^8$ CFU/day)	Mothers: not reported Children: from birth to 12 months of age	• All infants had low blood D-lactic acid levels (20 to 130 µM) • No significant difference in blood D-lactic acid levels between groups • No adverse events reported
Romeo *et al.*, 2006 The role of probiotics in the prevention of bacterial and Candida infections in Neonatal Intensive Care. Prospective study with control group.	Randomized controlled intervention	184 premature newborns in neonatal intensive care unit	1. *L. rhamnosus* GG ($3x10^9$ CFU/day) 2. *L. reuteri* ($1x10^8$ CFU/day)	28 days	• SS reduction in GI symptoms in *L. reuteri* group (*vs. L. rhamnosus* and placebo groups)
Betta *et al.*, 2007 Probiotics in the prevention of bacterial and Candida infections in newborns submitted to greater surgical interventions and admitted in NICU - retrospective group controlled study (abstract)	Placebo-controlled intervention [ABSTRACT ONLY]	24 newborns, mean gestational age: 36.4 ± 2.7 weeks, mean birth weight: 2568.3 ± 644 g	1. *L. reuteri* ATCC 55730 (dose not reported) 2. *L. rhamnosus* ATCC 53103 (dose not reported)	Not reported	• SS fewer episodes of infection in probiotic groups (*vs.* placebo group) • Safety data not presented

Table C-4 Oral Administration of *Lactobacillus reuteri* to Unhealthy or Diseased Infants

Title and Reference	Study Design	No. of Subjects	Probiotic Strain(s) [Dose; CFU/day]	Duration	Observations
Savino *et al.*, 2007 *Lactobacillus reuteri* (ATCC 55730) versus simethicone in the treatment of infantile colic: a prospective randomized study	Open-label randomized controlled intervention	90 breastfed colicky infants (54♂ and 39 ♀); age 11 to 80 days	*L. reuteri* ATCC 55730 (10^8 CFU/day in oil suspension) Simethicone (60 mg/day)	28 days	• No infants withdrew because of any adverse effects related to the trial • SS reduction in crying time in the *L. reuteri* group

SUBMISSION END